

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7:

(11) Internati nal Publicati n Number:

WO 00/32140

A61F 5/58, A01N 1/02

A1

(43) Internati nal Publication Date:

8 June 2000 (08.06.00)

(21) International Application Number:

PCT/US99/28408

(22) International Filing Date:

30 November 1999 (30.11.99)

(30) Priority Data:

09/201,594

30 November 1998 (30.11.98) US

(71) Applicant: IVF SCIENCES COLORADO, INC. [US/US]; Suite 300, 799 E. Hampden Avenue, Englewood, CO 80110 (US).

(72) Inventors: GARDNER, David, K.; 9927 Clyde Circle, Highlands Ranch, CO 80126 (US). LANE, Michelle; 1661 West Canal Circle, #324, Littleton, CO 80120 (US).

(74) Agents: FISCHMANN, Kent, A. et al.; Holme Roberts & Owen LLP, Suite 4100, 1700 Lincoln Street, Denver, CO 80203 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

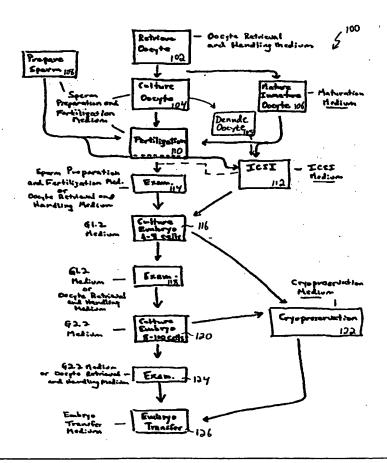
With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SYSTEM AND SEQUENTIAL CULTURE MEDIA FOR IN VITRO FERTILIZATION

(57) Abstract

Instead of immersing human reproductive cells in a single culture medium throughout the various procedures used in IVF, a process is provided by which the reproductive cells may be moved through a sequence of distinct culture media as the various IVF procedures are carried out. In one implementation, the culture media specifically formulated to provide a physical environment similar to that found within the female reproductive tract and conducive to growth and development of human reproductive cells during the various stages of the IVF process. In this regard, specifically formulated culture media can be applied to support the reproductive cells in one or more of the following procedures: oocyte retrieval and handling; oocyte maturation; ordinary fertilization; oocyte, zygote and embryo examination and biopsy; embryonic development to the eight-cell stage; embryonic development to the blastocyst stage; embryo transfer, and cryopreservation.



KONG TAMO AN SHOUTH

A series of the constitutes and the advanced as effecting an extensional to the control of th

THE PERSON OF THE BEST WELL AND THE STATE OF THE STATE OF

The control of the control of the control of the property of the control of the c

E and therefore the state became the result of the second of the state of the second o

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania Armenia 11 of the 150	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia profit and grot	Jenii ile	Medical and also state of the art	LT' &:	Lithuaria (1)	SK	Slovakia
AT	Austria	FT	Prance	1.11	Inventoure	CN	Camanal
AU	Australia Pote Glass of Ch	orii	Gal Day - they block in	LV ,	Latyia Control 1975, Principle Monaco	SZ	Sanziland
AZ	Azerbaijan	CR.	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GB	Georgia	MD	Republic of Moldova Wiadagascar	TG ,	Togo
BB	Barbados TIS AD A O	CH.	Ghast Surf Hard OJ, Cost.	MG!::: '	Wadagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Paso 20-3	CR	Giecepanio di sosotani	1- 77, 3	Wadagascar The former Yugoslav Republic of Macedonia Mali Mongolia	TALL	Turkey
BG	Bulgaria	HU-	Hangary	ML `	Mali -	TT	Trinidad and Tobago
BJ	Benin	IR A	- Heland Language constitution seem and the	MN .	Mongolia Mauritania	UA :	Ukraine State
BR	Brazil	IL.	Ishae 11	MR	Mauritania	UG	Uganda
BY CA	Belarus	IS	Iceland	MW,	Malawi Mexico Test and a state of	US	United States of America
CF	Canada 51311111 FOR	趣 51以	india - Telebos medide, c	MX DE	Mexico Para La	UZ · · ·	Uzbekistan
CG	Central African Republic	JP .	Japan:	NE	Niger	VN	Viet Nam
CH	Congo	KE	Kenva	NL.	Netherlands	YU :	Yugoslavia :
CI	Central African Republic Congo Switzerland Côte d'Ivoire	KG :	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CM	Côte d'Ivoire Cameroon (ASTE DE PRESIDE	KP rdz	Democratic People's	NZ	New Zealand	21.5	a morning and
CN	China		Republic of Korea	PL .	Poland		Fig. 1 Big 1194
CU	China Cuba	KR	Republic of Korea	PT	Portugal		
cz	Czech Republic 15710 (1.1.	K25 . 3	Resistant Colores of the	RO:	Romania (2) 261 1 11 2 Russian Federation		ನ್ನಡ್ಟ್ 'ಎ.
DE	Czech Kepublic	III.	Saint Lucia	RU	Russian Federation		
DK DK	December Control of Control	5 2000	Liectienstein	SO .	Sudan Sweden	T in	f ⁽¹⁾ = \$6.884.844
EE	Catania	Y.W	Sri Lanka				
E.E.	Estonia	LR	Liberia	SG	Singapore		

SYSTEM AND SEQUENTIAL CULTURE MEDIA FOR IN VITRO FERTILIZATION

FIELD OF THE INVENTION

The present invention relates generally to human in vitro fertilization (IVF) and, in particular, to a sequential culture media system and process to be used in oocyte retrieval, handling and maturation, sperm preparation, fertilization, embryo development and transfer, and cryopreservation. The invention provides the gametes, zygote and developing embryo with a physical environment adapted to their physiological needs, so supporting their normal growth and development in vitro and increasing the likelihood of successful pregnancy.

5

10

15

20

25

30

BACKGROUND OF THE INVENTION

In vitro fertilization seeks to duplicate, to a large extent, the conditions and processes normally occurring within the female reproductive system that are necessary to oocyte development, fertilization and early embryonic development. In the clinic and laboratory, IVF involves several discrete procedures, such as collection of the oocytes from the ovary of the mother, preparation of the sperm, fertilization, and, once fertilized eggs are identified, a period of early embryonic development, and then transfer of the embryo to the mother's uterus. Each of these steps can take place over extended periods of time, during which the individual cells involved have a continuing need for nutrients, and are subjected to significant stress as a result of clinical manipulation and changed environmental conditions.

During IVF, a culture medium is ordinarily used as a substitute for the fluid secreted by the female reproductive tract that would ordinarily surround the gametes, zygote, and developing embryo. Most laboratories carrying out IVF use a single culture medium throughout the various procedures involved. In a number of laboratories, there has been a tendency to use tissue culture media for IVF procedures, such as Ham's F-10, which is formulated to support somatic cell growth, not gamete or embryonic cell growth. Tissue culture media generally are complicated systems, containing an array of amino acids, vitamins and other constituents. They can contain components that significantly impair embryonic development and viability after transfer. Further, to the extent tissue culture media contain components that are

वर्षात्रक जुला

ge GF

913

1.5

U)

20

х.:

EG 17

generally needed by the gametes and the embryo, the media are not formulated to provide the components at levels appropriate to support healthy gamete and embryonic development.

5

10

15

20

25

30

Other laboratories have used simple culture media, consisting of balanced salt solutions supplemented with carbohydrate energy sources such as glucose, pyruvate and lactate. Examples include Earle's, T-6, and human tubal fluid (HTF). These media generally lack certain key components found in the female reproductive tract, such as non-essential amino acids, and their constituents are not formulated in concentrations that meet the specific changing needs of the gametes and developing embryo at various stages of their development.

The two types of culture media commonly used for IVF generally are only capable of supporting embryonic development to the eight-cell stage. Such media are ordinarily not capable of supporting and producing a viable blastocyst stage embryo, complete with an epithelium and competent inner cell mass. Accordingly, embryo transfer, the return of the fertilized oocyte to the uterus of the mother, usually occurs at around the four-cell stage (day two) or around the eight-cell stage (day three). This is a time when the four- or eight-cell embryo would not typically have arrived in the uterus of the mother, if fertilization had occurred in vivo. Embryo transfer at this time involves placing the cleavage stage embryo in an environment oriented to a blastocyst stage embryo. The cleavage stage embryo must then undergo further development in a non-homologous environment to reach the blastocyst stage, in which the embryo has trophectoderin cells capable of implanting in the uterine lining.

Recent research and human trials have led to the development of two new culture media, G1 and G2, which represent significant advancements in adaptation of culture media to the physiological needs of the cleavage stage embryo and the embryo in the eight-cell through blastocyst stage of development. These media are described in the following publications: Barnes, Crombie, Gardner, et al, Blastocyst Development and Birth After In-vitro Maturation of Human Primary Oocytes, Intracytoplasmic Sperm Injection and Assisted Hatching, Human Reproduction, vol. 10, no. 12, pp. 3243-47 (December, 1995); Gardner and Lane, Culture and Selection of Viable Blastocysts: A Feasible Proposition for Human IVF?, Human Reproduction Update, Vol. 3, No. 4, pp. 367-82 (1997); Gardner, Vella, Lane, et al, Culture and

Transfer of Human Blastocysts Increases Implantation Rates and Reduces the Need for Multiple Embryo Transfers, Fertility and Sterility, Vol. 69, No. 1, pp. 84-88 (January 1998). Use of these media, and particularly the G2 medium, supports the growth and development of viable blastocyst stage embryos in vitro. Accordingly, the development of these media paves the way for new approaches to embryo transfer to the uterus at the blastocyst stage, when the embryo is most adapted to surviving in the uterine environment and has developed structures and capabilities required for implantation to take place. Research utilizing the G1 and G2 media, and embryo transfer at the blastocyst stage, suggests that these media contribute to higher pregnancy rates, and reduces the need for transfer of multiple embryos and the risk of multiple births. Neither of these media, however, is optimized for supporting the gametes, oocyte maturation, or fertilization.

5

10

15

20

25

30

SUMMARY OF THE INVENTION (Section of the Victor of the Control of

the a growbout her galang and to widenon for vital visit

It has been recognized that IVF processes may be improved by providing specific media and media sequences for supporting gametes, zygotes and developing embryos relative to various phases of the IVF process. In certain respects, such media and sequences better reflect in vivo development. Within the female reproductive and sequences better reflect in vivo development. system, the oocyte is developed within and released from the ovary during ovulation, and proceeds through the oviduct towards the uterus. During this journey, it and the time of the experiences a dynamic physical environment. The fluid of the oxiduct contains a crown as number of components that provide nourishment to the oocyte and its surrounding good their of marks directly of cumulus cells, and that also appear to interact with the oocyte and its cumulus cells, so stimulating development. Similarly, the fluid of the female reproductive tracted in the stimulating development. provides nourishment to sperm traveling through the oviduct, and also stimulates even in and the transfer of the second of the certain changes in the sperm necessary to fertilization. Once fertilization occurs, the ಾರ್ ಸಹಾಸ್ ಕ್ಲೇಡ್ ಚಿತ್ರಗಳು resulting zygote travels down the oviduct and enters the uterus approximately three and enters the uterus approximately three L now the ser parameters of days later, undergoing internal transformation and experiencing a changing to account environment. the collection of the state of the section of the s

As the zygote travels, cell division, or cleavage, occurs as well as significant developmental changes. The cells of early embryonic development have different capabilities and nutritional needs from those of later embryonic development prior to a significant development development prior to a significant development develo

implantation. The zygote and cleavage stage embryo (up to the eight-cell stage) are characterized by low levels of biosynthesis, low respiratory rates, only limited ability to metabolize glucose, and a capacity to utilize pyruvate. As the embryo develops, and genome activation occurs, the embryo gains an increased capacity to utilize glucose. At the blastocyst stage of development, when the embryo is entering and within the uterus, the embryo's metabolic system has developed and the embryo has a substantially greater capacity to use and need for glucose, and less need for pyruvate. The makeup of the fluid surrounding the developing embryo in the female reproductive tract seems to be tailored to these changing needs: in the oviduct at the time when the oocyte and developing embryo are present, relatively low levels of glucose are found, while pyruvate concentrations are high; at the time the embryo enters the uterus, glucose reaches its highest level and the pyruvate concentration is comparatively low. Cleavage stage embryos, like the oocyte, are susceptible to loss of amino acids through their cell membranes when surrounded by an environment having a low concentration of such factors. Such loss of internal amino acids can have devastating effects. Again, as if in response to these needs of the osmolyte sensitive oocyte and cleavage stage embryo, the female reproductive tract typically has high levels of specific amino acids that are very similar to those found in the oocyte and cleavage stage embryo.

20

15

5

10

In view of the foregoing, an important object of the present invention is to further improve and enhance the culture of human reproductive cells in vitro. The invention is intended to promote the health and viability of the gametes, zygote and embryo at various stages of the IVF process, thereby improving the overall efficiency of the IVF process and increasing pregnancy rates.

25

30

In general, the present invention involves the application of separate media specifically formulated to meet the physiological needs of the gametes, zygote and/or developing embryo in various stages of their development, and to support the processes necessary to accomplish fertilization and embryonic development in vitro. The present invention also generally contemplates a sequential culture media system, in which the separate media utilized have integrated formulations, intended to minimize trauma to the reproductive cells as they are moved from one medium to another during the IVF process.

In one aspect of the present invention, an oocyte retrieval and handling medium is provided for use in the clinical procedure of retrieving the oocyte from the mother. The medium may be used for flushing, washing and holding the oocyte was during the process of removing the occyte from the mother's overy, and for storing the oocyte for a period prior to fertilization. An optional use of the medium envisioned by the invention is with procedures where handling or manipulating the oocyte, and the zygote, or embryo is necessary, such as examination of the oocyte to determine whether fertilization has occurred, or examining the embryo to determine the progression of its development. The present invention includes use of an oocyte retrieval and handling medium comprised of water, ionic constituents, and a buffer. Preferably the buffer used in the medium is 4-Morpholinepropanesulfonic acide with (MOPS) or N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES).: In the addition, the medium may be supplemented with the carbohydrates glucose alactate and pyruvate. The medium may be supplemented with non-essential amino acids. An optional formulation of the medium, lacking calcium and magnesium, may be used in biospsy procedures. Another optional formulation of the medium includes antibiotics, such as penicillin and/or streptomycin, to destroy bacteria that might be introduced into the medium during the process of oocyte collection. The process of oocyte collection.

31

• •

Ĺ

5

10

15

20

25

30

Another aspect of the present invention involves the provision and use of amonocyte maturation medium, for example, in circumstances where the occyte is a removed from the mother before it is mature. An example of a situation where application of this medium may be desired arises when it is necessary to treat the cocytes collected from the mother with hormones in vitro due to the mother's intolerance of such hormones. The invention contemplates holding the occytes in the maturation medium for a period following collection of the occytes to promote development prior to fertilization. An optional use of the maturation medium include accordance with the invention is for collection, although the most cost effective approach will normally involve use of the retrieval and handling medium for initial of the standard period prior to fertilization. The present invention contemplates use of a maturation medium comprised of water, ionic constituents, and a buffer.

lactate and pyruvate. Specific formulations in accordance with the present invention may involve successive supplementation of the medium with one or more of the following: non-essential amino acids; essential amino acids; cysteamine; human serum albumin (HSA) and hyaluronate; one or more growth factors such as insulin transferin selenium (ITS), insulin-like growth factor (IGF), and epidermal growth factor (EGF); and hormones follicule stimulating hormone (FSH) and human chorionic gonadotrophin (hCG).

Another aspect of the invention involves the provision and use of a sperm preparation and fertilization medium. This medium may be applied to wash, prepare, and store sperm, to store the oocyte in the period prior to fertilization, and to serve as the medium in which the sperm and oocyte are placed together and fertilization occurs. The present invention contemplates use of a sperm preparation and fertilization medium that includes water, ionic constituents, and a buffer. Preferably, the medium contains an elevated concentration of sodium, as compared to the oocyte retrieval and handling medium, to promote sperm function and fertilization. In addition, the medium may be supplemented with an elevated phosphate concentration, as compared to the oocyte retrieval and handling medium. Even more preferably the medium is supplemented with the carbohydrates glucose, lactate and pyruvate. Specific formulations may involve supplementation of the medium with one or more of: bicarbonate; glutathione to promote sperm head decondensation; non-essential amino acids; HSA and hyaluronate; and antibiotics such as penicillin and streptomycian ideas of beauty and the streptomycian ideas of the streptomycian id

sperm injection (ICSI) and related methodology. The ICSI procedure may be necessary where there are obstacles to normal fertilization, such as a thickened zona pellucida on the oocyte hindering sperm head penetration. ICSI involves removal of the cumulus cells and injection of the sperm into the oocyte, ordinarily through a glass pipette. The invention contemplates placing sperm in the ICSI medium, capturing the sperm by drawing the medium containing sperm into the pipette, inserting the pipette containing medium and sperm into the oocyte, and, following insertion into the oocyte, transferring the medium containing sperm from the pipette into the oocyte. The ICSI medium used in the present invention includes the constituents water, ionic

constituents and a buffer. Preferably, in the present invention the medium lacks phosphate. More preferably, the buffer used in the medium is MOPS or HEPES. Additionally, the medium may be supplemented with the carbohydrates lactate and pyruvate and the medium may be further supplemented with one or more of the non-essential acids most abundant in the oocyte: glutamine, glycine, proline, serine, and taurine. In one formulation, the ICSI medium used is supplemented with hyaluronate or polyvinylpyrolidone (PVP) to slow or immobilize the sperm so that they may be captured by pipette for the ICSI process. Further, an alternative formulation of the ICSI medium referred to as denuding medium used in the invention includes hyaluronidase, which is included in the portion of the medium used to denude the oocyte prior to the ICSI process.

5

10

15

20

25

30

Another aspect of the present invention involves the provision and use of a medium for supporting initial cell cleavage and embryonic development following fertilization, the medium herein referred to as G1.2. The invention contemplates washing the inseminated oocyte and zygote in the medium and placing the zygote in the medium for a period of about 48 hours to support cell cleavage and development through about the eight-cell stage. The present invention involves use of a medium that includes the constituents water, ionic constituents, and a buffer. Preferably, the medium is supplemented with the carbohydrates glucose, lactate, and pyruvate. The medium may also be supplemented with non-essential acids. Specific formulations in accordance with the invention may include one or more of the following supplements: EDTA; HSA; and hyaluronate. The form of glutamine used in the medium is a supplemented breakdown to the waste product ammonium, which is particularly stable and less prone to breakdown

A further aspect of the invention involves the provision and use of a second medium for embryo development, herein referred to as G2.2. The invention contemplates placing the embryo in the G2.2 medium for a period of about 48 hours, or preferably at or after the eight-cell stage, and continuing through the blastocyst stage of development and up to the point of embryo transfer. This medium is specifically adapted for and has as its preferred use support of the embryo from the eight-cell stage through the time at which implantation occurs, in tandem with the use of G1.2003 for initial embryonic development. The invention involves a G2:2 medium that

? ;

43.5

includes water, ionic constituents, and a buffer. Preferably the medium is supplemented with the carbohydrates glucose, lactate and pyruvate. More preferably, as compared to medium G1.2, medium G2.2 is supplemented with depressed levels of lactate and pyruvate, and elevated levels of glucose. Additionally, the medium may be supplemented with the non-essential amino acids, except taurine. Specific formulations in accordance with the present invention involve supplementing the medium with one or more of: essential amino acids, which stimulate development of the inner cell mass of the blastocyst; vitamins, which further facilitate the function of the blastocyst; HSA; and hyaluronate. An important aspect of the G2.2 medium, in all formulations, is the absence of EDTA.

5

10

15

20

25

30

Another aspect of the invention is the provision and use of an embryo transfer medium. The invention contemplates that this medium will be used as a carrier for the embryo when it is transferred back into the mother. The invention may involve the same formulations of the medium for embryo transfer as are used with medium G2.2. More preferably for embryo transfer, however, the formulation of G2.2 is supplemented with a higher concentration of hyaluronate, which supports implantation of the embryo in the mother's uterus.

A further aspect of the invention is the provision and use of a medium for cryopreservation of the embryo and/or oocyte. The invention contemplates that the embryo may be placed in the medium at either the one- to eight-cell stage or eight-cell to blastocyst stage, and then frozen and stored in the medium. The invention also contemplates the che medium may be used for cryopreservation of the oocyte. The cryopreservation medium contains ionic constituents, and a buffer. Preferably, it contains the MOPS or HEPES buffer. More preferably, it contains the carbohydrates lactate, pyruvate and glucose. Even more preferably, it contains HSA. Most preferably, the medium contains certain additives such as glycerol, ethylene glygol, DMSO, and/or sucrose.

According to a further aspect of the invention, different media are used for two different phases of the IVF process, such as oocyte collection and maturation, sperm preparation, fertilization, embryo development and/or embryo transfer. One associated process involves obtaining a gamete from a first medium and introducing the gamete into a second medium different from the first medium, wherein

٠,٠

fertilization occurs in the second medium. The step of obtaining a gamete from a first medium may include extracting an oocyte from an oocyte collection medium or oocyte maturation medium as described above. Additionally or alternatively, the step of obtaining may involve extracting sperm from a sperm preparation and fertilization medium as described above which, in turn, may be different from the oocyte medium. The step of introducing the gamete into the second medium may involve introducing the sperm and/or oocyte into a fertilization medium, or injecting sperm into an oocyte contained in the second medium. The various media may have integrated formulations for minimizing trauma to the reproductive cells.

5

10

15

20

25

30

Another associated process in accordance with the present invention involves obtaining a zygote or embryo from a first medium wherein fertilization has occurred and introducing it into a second medium different from the first medium for a first growth phase. The first medium may be a fertilization medium as described above and the second medium may be the G1.2 medium as described above. The second medium may be used for supporting initial cell cleavage and embryonic development. The method may further involve transferring the resulting embryo from the second medium to a third medium for a second growth phase. The third medium may be a second G2.2 medium as described above.

A further associated process in accordance with the present invention involves obtaining an embryo from a first medium and introducing the embryo into a second medium different from the first medium for transfer of the embryo into the mother for implantation. The first medium may be a G2.2 medium as described above and the second medium may be an embryo transfer medium as described above.

BRIEF DESCRIPTION OF THE DRAWING to bus emerged, estates!

positions and MAPS of HEFUE buffer (b) to profession on singless or or other constitues

 $\mathcal{E}^{(i)}$

For a more complete understanding of the present invention and further complete understanding of the present invention and the present inven

in conjunction with the drawings, in which: Also the loss of the conjunction with the drawings of which the drawings of the loss of the lo

Figure 1 is a flowchart illustrating an IVF process in accordance with the accordance present invention.

DETAILED DESCRIPTION OF THE INVENTION OF A SECOND SECOND

privationally that there is a truly doing of Destrictioners account in accurate

The following description discloses the composition of various culture media in accordance with the present invention that are particularly adapted for use with IVF. Each of these media is specifically formulated to meet the physiological needs of the gametes, zygote and developing embryo at key points in the reproductive process. Also disclosed is a sequential culture media system. While each of the separate media could be used independently, the media also may be formulated together as a system, sharing a core group of ionic and non-essential amino acid constituents, with the objective of minimizing trauma to the oocyte, and the resulting zygote and embryo, as they are moved from one medium to another. The following description also discloses methods of using the media and the sequential culture media system in various clinical and laboratory procedures by which IVF is carried out, as well as methods of making the culture media.

A. Composition of the Sequential Culture Media

1 (

1. Oocyte Retrieval and Handling Medium

A preferred oocyte retrieval and handling medium is an aqueous solution comprised of the ionic components sodium, potassium, phosphate, magnesium, bicarbonate, and calcium, to maintain an osmotic environment that does not stress the oocyte, and a buffering system, preferably MOPS or HEPES, to maintain the pH of the medium within the physiological range of 7.3 to 7.4. The ionic components are

10 Clarical terminal area of the control of their new fundaments the resulting in express as

In the filter area of the factor of the courtes of the condition of this section area of the filter product of the product of the product of the courtes of the courtes of the filter of the product of the courtes of t

included in the preferred amounts depicted in column A of Table 1, and may be included in amounts described in the ranges depicted in column B of Table 1.

Table 1

Composition of Oocyte Retrieval and Handling Medium*

Component of the state of the particular Alteral both stocks	The Property of the B
Most Preferred	Preferred
Kul har say at framissant at for on, and a said a district year.	35-75
MgSO ₄ .7H ₂ O 1 to the man of the mount of and the state of the state	9.2 ± 4.0 2 0 = 10 0
CaCl ₂ .2H ₂ O	y 1000-100 pg 60.8, - 2.8
NaLactate (L-isomer) 10.5 NaPyrovate 0.32	5.0 - 20.0 0.1 - 1.0
Alanine (ala) 0.1 Asparate (asp) 0.1 Asparagine (asn) 0.1 Glutamate (glu) 0.1 Alanyl - Glutamine (ala - gln) 25 0.5 Glycine (gly) 0.1 Proline (pro) 0.1	0.01 - 0.5 0.01 - 0.5 0.01 - 0.5 0.01 - 0.5 0.01 - 0.5 0.01 - 0.5 0.01 - 0.5
	Most Preferred Concentration NaCl KCl NaH ₂ PO ₄ .2H ₂ O MgSO ₄ .7H ₂ O NaHCO ₃ MOPS / HEPES CaCl ₂ .2H ₂ O Glucose NaLactate (L-isomer) Alanine (ala) Asparate Asparate Asparate (asp) Asparagine (asn) Glutamate (glu) Alanyl - Glutamine (gly) Proline (gly) Proline (ser) NaCl Sonce The Concentration 90.08 5.5 1. A value of the Concentration 90.08 5.5 1. A value of the Concentration 90.08 1. A value

* Concentrations are in millimoles unless otherwise indicated; the medium is aqueous.

30

35

It should be noted that Table 1 and the other tables presented in this section also describe the preferred form of the components used to make the respective culture media in practice. The MOPS buffer has not been used before in IVF procedures, and is preferred because it is not known to exhibit any toxic effects to reproductive cells and does not require maintenance of a CO₂ atmosphere above the medium. HEPES may also be utilized, although some research indicates a possible toxicity to reproductive cells. Table 1 depicts the preferred amount and ranges for the MOPS or HEPES buffer, although other buffering systems might be used. For example, a bicarbonate buffering system may be used because it is compatible with human

reproductive cells. Such a system would not ordinarily be practical with oocyte collection, because it requires the maintenance of elevated levels of CO₂ in the atmosphere surrounding the medium, which is ordinarily accomplished by use of a gassing incubator system that maintains a 3%-10% CO₂ atmosphere. Oocyte collection is a clinical procedure, in which it is typically not possible to maintain an elevated CO₂ atmosphere. In some clinical environments, such as where a humidicrib is available, it may be possible to perform oocyte collection in an elevated CO₂ atmosphere, and a bicarbonate buffer accordingly may be used. In accordance with the present invention, any buffering system used preferably maintains its buffering qualities during exposure of the medium to the atmosphere, and as well is preferably compatible with and not toxic to human reproductive cells.

The oocyte retrieval and handling medium also includes the carbohydrates glucose, lactate, and pyruvate, at levels similar to those found in the female reproductive tract at the corresponding point of ovulation. The preferred amounts and ranges in which these are found in the medium are depicted in Table 1. In addition, the preferred medium contains Eagle's non-essential amino acids (i.e., those not required for the development of somatic cells in culture) alanine, aspartate, asparagine, glutamate, glycine, proline, serine, and taurine, plus glutamine in the form of alanyl-glutamine, at levels similar to those found in the female reproductive system and in the oocyte. The preferred amounts and ranges are depicted in Table 1. The inclusion of non-essential amino acids and alanyl-glutamine in the medium is important to preventing osmotic shock; a medium lacking these components may drain the oocyte of its internal pool of amino acids, resulting in considerable intracellular trauma. An optional formulation of the medium which may be used in biopsy procedures, omits calcium and magnesium. Another optional formulation of the medium may include one or more antibiotics, such as penicillin and streptomycin, to destroy any bacteria that might be present around the oocyte or that might be introduced through the clinical procedure of oocyte removal.

2. Oocyte Maturation Medium

5

10

15

20

25

30

The oocyte maturation medium is adapted for use with immature oocytes. Oocyte maturation is typically used with mothers who are unable to withstand the

hormonal treatment ordinarily employed in IVF. Oocyte maturation generally involves treating the immature oocytes in vitro with the hormones follicle stimulating hormone (FSH) and human chorionic gonadotrophin (hCG) rather than injecting these hormones into the mother. The preferred medium is an aqueous solution that contains ionic constituents similar to those used in the oocyte retrieval and handling medium, at similar concentrations, although the magnesium level is increased and the calcium level decreased to maintain a 2:1 magnesium to calcium concentration. A buffer is included in the preferred medium to maintain a physiological pH. Because oocyte maturation ordinarily occurs in an incubator or isolette in which an elevated CO₂ atmosphere can be maintained, a bicarbonate buffering system is preferred. Other buffers may be used, provided they are compatible with the oocyteland other components of the medium. Table 2 provides the most preferred amounts of each of these components, as well as the preferred ranges of these components.

5

10

The second of the content of the configuration of t

grafig al jakuwasi innani? - S

€. €

. . . .

25

Country over the state of the country of the countr

and the first case of the contract of Table 2-1075 for the first the second

	Composition of Oocyte Maturation Medium*	• "
	Component	. <u>B</u>
	mis rievei el municipas electricia electricia el Mist Preferred	Preferred
	de la tras pre abies quires les comes en la Concentration de la come de	Rang
	N. C.	
5	NaCl Company of the participation of the solution of the solut	80.0 - 100
	3.3	3.5 - 7.5
	NaH ₂ PO _{4.2} H ₂ O Contract of the value of the contract of th	0.05 - 1.5
	NaHCO3 , the two Attentions of the control of the second of the control of the co	0.2 - 4.0
	CaCl ₂ 2H ₂ O	15 - 30.0
10	Glucose (1, 2011) englicoste o probabilità de la company de la 15 company de la company de la la company de la com	0 _: 8 - 2.8 0.5 - 5.5
	NaLactate (L-isomer) 5.87	2.0 - 20.0
	Naryruvate 0.1	0.01 1.0
	Alanine Asparate Suggest of modern production of the transfer of the control of t	0.01 - 0.5
1.5	0,1	0.01 - 0.5
15	Asparagine The County of the state of the st	0.01 - 0.5
	Glutamate O.1 Alanyl - Glutamine of other and the street will be a set of the street	0.01 - 0.5
	Glycine	0.01 - 2.0
	Proline Destriction Vision Program Cold and Time 6.17 (Bell of the Cold of the	0.01 - 0.5
20	0.1	0.01 - 0.5
	Cysteamine with the property of the second to the property of the second to the second	0.01 - 0.5
	IArginine-HCI	0.1 - 2.0
	I -Cystine 2HCl	0.1 - 1.2
	L-Histidine-HCl-H2O 0.2	0.05 - 0.25 0.1 - 0.4
25	L-Isoleucine 0.4	0.1 - 0.4
	- L-Leucine	0.1 - 0.8
	L-Lysine-HCl L-Methionine of the first of the state of th	0.1 - 0.8
	L-Methionine Co. 13 Provide Co. 1	0.05 - 0.25
30		0.i - 0.4
	T. (2)	0.1 - 0.8
		0.1 - 0.9
	L-Valina to the section of the secti	0.1 - 0.4
	$\mathbf{p} \cdot \mathbf{p} = \mathbf{p} \cdot \mathbf{p}$	0.1 - 0.8
35	Choline Chloride is routed gradus rile and the stable of the control of the contr	.003 - 0.004 .003 - 0.01
	Folic Acid i-Inositof After the forest before the total and the collection at the collection of the collection and the collecti	
	Niacinamide (C. zamari (& E) 1. zadaj 1 ora o i jazanti 2 0.0032 – na en zeud samite o 0.0000 Pyridoxal HCl	004 - 0.016
40	Pyridoxal HCl 0.0049	.002 - 0.01
40	Richard Land Land Company of St. 1980000 Page 1992 of Land Company of Company	กดิ์ 1 2 ก กกกล
	Thiamine HCl 0.003. Remains a construction of a gody hoge means on a class was solved one and entire course.	001 - 0.006
	HSA	
	HSA Hyaluronate on politicities invendent quality of the control	F - 10.0
	U.25mg/mi	0.05 - 0.5
	ITS PRODUCTION TO THE PRODUCT OF A SECOND TO S	
45	IGF4 they constructed they were an in the 100 to 100 mg/mile the second section of the	1 - 100
	EGF 100ng/ml	10 - 1000
	Toong/ml Compare halance as the file of the last of t	
	FSII Lorenz iller a ward more thought a few a foregone. The envelope of the land of the second of the land of the	0.01 - 10
	* Concentrations are in millimoles, unless otherwise indicated; the medium is aqueous.	D.01:⊱ 10
	constitutions are in minimoles, unless otherwise indicated; the medium is aqueous.	

The carbohydrates glucose, lactate and pyruvate are also included in the preferred maturation medium. Because of the presence and importance of cumulus cells that surround the developing oocyte, the glucose, lactate and pyruvate levels are adapted to the needs of the cumulus cells. Non-essential amino acids are preferably included in the medium to provide nutrients and avoid subjecting the oocyte to osmotic stress. Essential amino acids and vitamins may also be included to provide nutrients to the cumulus cells. The medium also contains HSA and hyaluronate, The medium also contains HSA and hya which act as a source of macromolecules. Insulin transferin selenium (ITS), insulinlike growth factor (IGF), and epidermal growth factor (EGF) are included to support the function of cumulus cells, which, in turn, nourish and stimulate the oocyte. FSH and hCG are added to stimulate the cumulus and oocyte to undergo changes ina dak r associated in vivo with ovulation. It should be noted that, when the maturation of the same associated in vivo with ovulation. medium is prepared, ITS, IGF, EGF and FSH and hCG are preferably the last-added ingredients. The preferred amounts and ranges of these components are found in any contract of the Table 2.

1,5

 $\langle j \rangle$

3. Sperm Preparation and Fertilization Medium

5.

10 ...

15

20

25

30

1-15

Current methods of in vitro fertilization employ the same medium for sperm preparation and fertilization as is used for embryo development. No attempt has been made to develop a separate medium for preparation of sperm that is also suitable for storage and support of the oocyte, for promoting the process of fertilization, and for supporting the zygotes formed when fertilization occurs. In many laboratories, the fertilization process is allowed to take place over an extended period which ranges from two to three hours to up to about sixteen (16) to eighteen (18) hours. During this time, the oocyte, sperm, and zygotes produced have significant nutritional needs. In addition, sperm function and fertilization tend to be encouraged when the surrounding fluid contains certain constituents. The sperm preparation and fertilization medium of the present invention is formulated to meet these concerns.

A preferred sperm preparation and fertilization medium in accordance with this invention has virtually the same composition of ions and non-essential amino acids as the oocyte retrieval and handling medium. The fact that these media share a similar ionic and amino acid composition minimizes the stress experienced by the

oocyte when it is removed from the retrieval and handling medium and placed in sperm preparation medium. Table 3 sets out the preferred amounts and ranges of the ionic and non-essential acid components.

FIG. I Substitute of the termination of the second state of the second state of the second se

_	an carcum guidhead bach a contribut Táble3 the de total and a contribute
5	Composition of Sperm Preparation and Fertilization Medium*
	A
	Bond of getterring of the section of Most Preferred and A state of Preferred
	Range
	NaCliques de l'ambiença de les activit activit de 100 pers à 200 de la compans de 75-100
	77.70 · / / / / / / / / / / / / / / / / / /
	NaH ₂ PO4.2H2O schools of the form of the second different in 5.5 and the carried second of 3.5 - 7.5 and the carried second of the second of
10	MgSO4.7H2O 10 10 10 10 10 10 10 10 10 10 10 10 10
	Olicose NaLactate (L-isomer) 0.2 - 4.0 0.5 - 5.6
	NaLactate (L-isomer)
	NaPyruvates is sure to success to the property of the property
	NaHCO3
15	CaCla attack
1.5	CaCl2:2H2O train office substitution to the country 1.8 of the substitution is 1.8 - 2.8
	Children to a pair water common with the edge of a recognition of the contract
	0.5 - 5.0
	* · · · · · · · · · · · · · · · · · · ·
	Alanine Asparate Other properties of the control o
	Asparagine side: a constitute to the constitute of the constitute
20	Glutamata 0.01 - 0.3
	Glycine strangage wester what the strangage of the strang
	Proline 0.1 0.01 - 0.5
	Carino U.UI - U.J
	Taurine 0.1 0.01 - 0.5
	ment reduced the light of the second of legal to accomplish the property of the second training of the second training of the second of the se
25	risa 5mg/ml
	Hyaluronate 0.1 mg/ml Penicillin was described as a substitute of the substitute o
	Penicillin standarto, areaso sal gribarature acti 0.00mg/ml areas a area cabara 0.02 - 0.5
	Streptomycin (C elychologic behavioral ad year are 0.05mg/ml and the form manifest 0.0110
	to extense and other property of the second

* Concentrations are in millimoles unless otherwise indicated; the medium is aqueous. Selected histographical empired base officience of a most flow our like of the self-equip. As than

30

As will be seen, the sperm preparation medium contains sodium at a higher concentration than the level found in the oocyte retrieval and handling medium. This elevated concentration of sodium promotes sperm function and fertilization, without causing undue osmotic stress to the oocyte. There is also a higher concentration of

phosphate, as compared to the occyte retrieval and handling medium. The glucose concentration of the sperm preparation and fertilization medium is elevated over that of the oocyte retrieval and handling medium, because glucose is the primary nutrient for sperm and cumulus cells around the egg. The lactate concentration of the present medium is lower than that found in the occyte retrieval and handling medium, to compensate for the tendency of sperm cells and cumulus cells to give off lactic acid as a waste product. A buffering system is used to maintain the physiological pH, and because sperm preparation and fertilization largely occur within an incubator that can maintain an elevated CO₂ atmosphere, a bicarbonate buffer is preferred. Glutathione (not present in the oocyte retrieval and handling medium) is included, to assist in the process of sperm head decondensation. Alanyl-glutamine (present in the oocyteretrieval and handling medium) is omitted from the present medium because it can impair sperm function and reduce fertilization. The same is true of the chelating agent EDTA, which (as will be discussed later) is present in the embryo development media. HSA, the most abundant macromolecule in the Fallopian tube and uterus, is included to support sperm and embryo function. Hyaluronate, which promotes sperm motility, and works in tandem with HSA, is also included. Because sperm tends to contain high levels of bacteria, one or more antibiotic substances are also included Penicillin, streptomycin, and/or gentamycin are preferred antibiotics. Table 3 sets out. the preferred amounts and ranges for these various components.

\$;;

ξ.

63

4. The ICSI Medium ...

5

10

15

20

30

15.5 10.0

In circumstances where it is desired to accomplish fertilization by other than natural interaction of sperm and oocyte, such as where the sperm is unable to fertilize the oocyte due to a thickened zona pellucida surrounding the oocyte, or where the cases 25 c sperm is from a male-factor patient, the sperm may be transported into the oocyte by a technique called intracytoplasmic sperm injection (ICSI). When the ICSI technique is * used, the cumulus cells are removed from the oocyte, and sperm is injected into the oocyte's interior using a glass pipette. The present invention contemplates use of a representation of the present invention contemplates use of a representation of the present invention contemplates use of a representation of the present invention contemplates use of a representation of the present invention of the pre single medium to bathe the occyte and also to serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the sperm as it is a serve as a carrier for the specimen as a serve as a s transported by injection into the oocyte. The medium, accordingly, is preferably at wolfe highly compatible with the interior and exterior of the oocyte. The ionic constituents

in the preferred medium are similar to those found in the oocyte retrieval and handling medium, except that phosphate is omitted, to avoid metabolic and homeostatic stress, and the magnesium-to-calcium ratio is 2:1. This ratio of magnesium to calcium is felt to be highly beneficial to the oocyte. Because ICSI is a clinical procedure performed outside the incubator, a buffering system that is effective in a normal atmosphere is used. MOPS and HEPES are accordingly preferred buffers for this medium. Because the cumulus cells have been removed from the oocyte, and the sperm is at the conclusion of its independent life, glucose, the main energy source for cumulus cells and sperm (but not the oocyte) is omitted from the medium. Pyruvate and lactate levels are increased, as these are a primary energy source for the oocyte. Only the non-essential amino acids most abundant in the oocyte - glycine, proline, serine and taurine - and glutamine (in the stable form alanyl-glutamine) are retained in the medium to avoid osmotic and pH stress and to nourish the oocyte. Preferably, the ICSI medium also includes hyaluronate or polyvinylpyrollidone (PVP), to immobilize or slow the sperm so that they may be captured in the ICSI pipette. Table 4 sets out the preferred amounts and the ranges of these components in the ICSI medium. Moreover, an alternative formulation of the ICSI medium includes hyaluronidase, which alternative formulation is used to pretreat the oocyte, to break down the hyaluronate gel holding the cumulus cells around the oocyte. This medium is referred to above as denuding medium, and lacks hyaluronate and PVP but includes hyaluronidase. The composition of the denuding medium includes the constituents of the ICSI medium (except hyaluronate and PVP) in the preferred amounts and ranges shown in Table 4 plus hyaluronidase in a preferred about of 40 IU/ml and a preferred range of Oct-80. Optionally, HSA may be included in the denuding medium in the preferred amount of 5mM and the preferred range of 1.0 - 10mM. than a soprious on his received is the property of the order of the order of

5

10

15

20

25

The substituted is seen maties as bornels are not given only to the configuration of the conf

įt.

 $\{...\}$

	general transport of the property of Table 4 and or research between your first
	Composition of Medium ICSI*
	Most Preferred services and Preferred services and Preferred
	Concentrati n Range
	NaCl
5	KCl MgSO ₄ 7H ₂ O ₃ 3.5 - 7.5 0.4 - 4
	MgSO ₄ .7H ₂ O ₃
	NaHCO ₃ 5 2.0 - 10. MOPS / HEPES 10 10 10 10 10 10 10 10 10 10 10 10 10
	the second secon
٠	$\begin{array}{cccccccccccccccccccccccccccccccccccc$
10	CaCl ₂ .2H ₂ O. 1 0.5 - 2.0 Not extend of increase in past sometime regression and the state of the second of of the sec
10	NaLactate (L-isomer) and the post of the control of the less of the NaPyruvate 0.32 0.1 - 1.0
	NaPyruvate 0.32 0.1 - 1.0
	erg i len ege i i literació i de l'appenda para sescre de loca trivocado i locas en marines. I
	Alanyl - Glutamine 0.1 - 2.0
	Glycine 0.1 0.05 - 2.0
15	Profine Serine 0.1 0.05 - 2.0 0.05 - 2.0
	Taurine 100 100 100 100 100 100 100 100 100 10
	HSA The state of 5 of the state of 5 mg/ml achieves solven for the state of the sta
	Hyaluronate 0.1mg/ml 200 2002-0.500 PVP 10% 1-20%
	PVP 10% 1-20
20	* Concentrations are in millimoles unless otherwise indicated; the medium is the standard of the standar
	adueous, Targe Many and Target an
	1 June 1 de la la la desta de la completa del completa de la completa de la completa del completa de la completa del la completa de la completa del la completa de la completa del la completa de la completa de la completa del la com
	5. Embryonic Development Medium G1.2. The present invention includes an embryonic development medium G1.2. The preferred application of this medium is to support development of the early one-to-
	preferred application of this medium is to support development of the early one-to-
25	eight cell embryo. As depicted in Table 5, the preferred medium has a backbone of
	ionic constituents and non-essential amino acids that is similar to that found in the
	oocyte retrieval and handling medium. Unlike the oocyte retrieval and handling
	medium, the G1.2 medium contains the component EDTA, which supports embryonic
	development and is believed to bind and disable toxins that might have a deleterious
30	effect on the early embryo, and which also suppresses glycolysis. In addition, this
	,

medium includes HSA and hyaluronate, in concentrations that are thought to support early embryonic development.

The preferred formulation of medium G1.2 differs from the previously published medium G1 in several important respects. First, research has shown that an 5 elevated phosphate concentration may not provide optimal conditions for growth of the developing embryo. Accordingly, the phosphate concentration has been decreased. Second, hyaluronate has been added to work in tandem with HSA. Third, alanyl-glutamine has been substituted for glutamine. A significant problem for embryo culture with amino acids is the natural decomposition of amino acids to ammonium, which decomposition is accelerated at higher temperatures such as the physiological temperature (37 degrees Celsius) used in IVF procedures. Ammonium can be toxic to embryos. Moreover, glutamine is especially prone to decomposition to ammonium within solution. Since embryos are generally cultured in medium GI or G1.2 for an extended period of up to about 48 hours, a significant quantity of ammonium can develop in the medium and be a significant inhibitor to embryo 15*:* development. Accordingly, the use of alanyl-glutamine provides substantial advantages; it is a particularly stable form of glutamine and is not prone to breaking down in solution. Also, the concentration of alanyl-glutamine in G1.2 has been reduced to .5 mM. These three modifications make G1.2 a significantly improved 20 medium for early embryonic development over medium G1. The most preferred amounts and preferred ranges of the components of medium G1.2 are depicted in Table 5.

e consections in the molfree for an loss of services is stronged; the modified to

S. D. on it also more assentances.

70.000

31 C 12 . 5

The last and Couries the Prominist result to the projection of continuous of the State and Couries and

au Mar caset Chours our survivious settige Pour Caster out ration at a said in the first

30

Table 5 and the Administration in the contract

	1, 10 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	Composition of Medium G 1.2*	
	Comp nent	Δ	D
	$c = m_1 \sin m_2$	Most Preferred at borros	enq and Preferred
	Charles Charles In his	Concentration 1. 10 pm	Story in the Range
5	NaCl Walsh	நாகத்த அக்க நகரு 90.08 முறையை கச	iguade <i>into</i> 80!0 - 100
	NaH ₂ PO ₄ .2H ₂ Octobro	$rac{5.5}{0.25}$ and $rac{6.5}{0.25}$ and $rac{6.5}{0.25}$	0.05 - 1.5
	MgSO ₄ .7H ₂ O	ra, A. Aris, Les Doubles no confision amb Agricultur 25	11.02 - 12.0 1 0.2 - 2.0
		25 Imagra e - Irrimote gin i bo Pakote naud util a	
	CaCl ₂ .2H ₂ O (Quarter to)	who set a act. 8.1. a set man for consuming	ombasi /
10	Glucose Judi 2440 Ang	त्तव प्रवासीता है । केंद्रावय के अने यो <mark>0.5</mark> में के कृतकार के हैं	(N. 1984) 1730158 (N. 1984) 1884
	NaLactate (L-isome)	2 TVI if Deal (2006) 200405. If containing the	. 1 lisot and 0 te 5:00 - 20.
	NaPyruvate	0.32 जुल्ली डॉडल १५७ हो। असंदारों में सुनम्मदल भीने उद्दर्शन	, 61 moored 0.1 - 1.0
	Alanine	and the second s	20 00 00 00 00 00 00 00 00 00 00 00 00 0
	Asparate Asparate	r. C. to regime so video e 2011 the malor video.	0.01 - 0.5
15	Acnoragina a see M	The face of the tender of the first posturation	0 ne 10 a 8 a 2 a 3
	Glutamate .	(1) 1. (1) 1. (1) (1) (1) (1) (1) (1) (1) (1) (1) (1)	non andin 0.010- 0.5%
	Alanyl - Glutamine		0.1 - 1.0
	Proline	0.1	0.01 - 0.5
20	Serine Serine	et est ett ett (2000-201) fællikkeit.	0.01 - 0.5
•	Taurine to the Last of the	enitant, perme or 0.1 or selection of the contract of the cont	0.01 - 10.0
	bacoqui I niu	ें हुई, रें के रूप के रेंट्रिक अपने अधिकर वहीं के	And Cost pappings
		employeest desp <mark>t.60</mark> 00000000000000000000000000000000000	
	Bi betail our on 12.15	ierred mages of the compens of the Euglid	sen ben steadern)
	HSA Hyaluronate	5mg/ml 0.1 mg/ml	1 - 10.0 0.02 - 0.5
		Č	

* Concentrations are in millimoles unless otherwise indicated; the medium is aqueous.

6. Embryonic Development Medium G2.2

Medium G2.2 is also formulated to support embryonic development. Its preferred use is with embryos from the eight-cell to the blastocyst stage (around 100 cells) to around one-hundred cell stage. The backbone of ionic constituents and non-essential amino acids preferably found in medium G2.2 is essentially the same as used with medium G1.2, except that the concentration of alanyl-glutamine has been increased. This reduces the risk of subjecting the embryo to osmotic stress as it is

moved from medium G1.2 to medium G2.2. Taurine is omitted because its benefits to the embryo appear to be confined to the period prior to compaction. Glucose, lactate and pyruvate are included as carbohydrates, except that the concentration of glucose is increased, while lactate and pyruvate are decreased, as compared to medium G1.2.

This modification in carbohydrate levels is in response to the increasing ability of the

This modification in carbohydrate levels is in response to the increasing ability of the developing embryo to metabolize glucose as an energy source, and reflects also the observed composition of the female reproductive tract. Eagle's essential amino acids are included in medium G2.2 because they are necessary to stimulate the growth of the inner-cell mass of the blastocyst. Vitamins are added as a group because in

animal studies they tend to facilitate the function of the blastocyst, including fluid accumulation in the cavity of the blastocyst. Importantly, this medium lacks EDTA.

The preferred amounts and ranges of the components of medium G2.2 are depicted in Table 6.

State of the

20 700

Table 6

, F.

	8 387 DW	colf the officient and fill of a	
15		Composition of Media	<u>ım G 2.2</u> *
	<u>Component</u>	A	<u>B</u>
		Most Pref	<u>Preferred</u>
	•	Concentr	ation
	bo consider	it vies seite such in	ation Range
	NaClossof wallo so	ilianty somoo militiista ima ili 19 0.08	3 m file (441 m) 102 (4800 - 100
	12.01		
	NaH2PO4.2H2O	# aptroduct from 2 2201 \$100.72 201 0.25	. papar vivo or Letter Lifeting 3.5 - 7.5 0.05 - 1.5
20	MgSO4.7H2O	groom a op die verratiide fan en 11e.	21 mg. half significant not 10.2 24.0
	37.77000	•	0.2 1.0
	CaCl2.2H2O ^{CT}	राज्य श्रोत्या तक्ष्या । रीते । राज्याय व्यक्ति	160-4 CORRUGE 374 20 15 - 30.0 0.8 - 2.8
			em eft redeau for the line for 0.5 - 5.5
	NaLactate (L-isom	er) 5.87	0.3 - 3.3
25	NaPyruvate	the purbased unional and think	**************************************
	Alanine	T.O. of the control of the Falsa C.	0.01 - 1.0 0.01 - 0.5
	Asparate	0.1	
	Asparagine	0.1	0.01 - 0.5
	Glutamate .		0.01 - 0.5
30		0.1	0.01 - 0.5
50	Glycine	uliu Makenwika, ani di malika	
	Proline	0.1	**************************************
		1 A 34	0.01 - 0.5
	Serine	67 miles & 1/2 0.1	0.01 - 0.5
25	L-Arginine-HCl	0.6	0.1 - 1.2
35	O'. L-Cystine 2HCl	30° 6° 5° 0.1	0.05 - 0.25

PCT/US99/28408 WO 00/32140

9	L-Histidine-HCl-H2O	with the state 0.2 In Fig. 2 members	0.1 - 0.4
	L-Isoleucine	0.4	0.1 - 0.8
•	L-Leucine	in b 12 49 - 0.4 一ついかから g 0.4	0.1 - 0.8
	L-Lysine-HClg of the second of the	0.4 Agonim szere y 0.4 r. mitobinisal ora	ोव व्याप्त 0.1 - 0.8
5	L-Methionine	and the state of 0.1 way from the color and with	0.05 - 0.25
	L-Phenylalanine	0.2	0.1 - 0.4
	L-Threonine and the safety care	v _i ta 、 が 初かる 0.4 m(Aydouther to 40)抗	0.1 - 0.8
	L-Tryptophan	79 6 10 00 00 00 00 00 00 00 00 00 00 00 00	0.1 - 0.9
	L-Tyrosine 2Na	0.2	0.1 - 0.4
10	L-Valine of the state state of the	ভাল িক্ষাভা (ŏ.4 ৭€2 কালিকৈ কল্যাঃ সঞ্	500 0 0.1 ² 0.8
	D-Ca Pantothenate	0.002 (C. 10)	· 0.001,- 0.004
	Choline Chloride	0.007	0.003 - 0.01
	Folic Acid services and services	The target of the same of the same of	0.001 - 0.0045
	i-Inositol	0.0023 0.0111 of he second s 0.0082	0.005 - 0.02
15	Niacinamide Contract Williams	0.0082	0.004 - 0.016
	Pyridoxal HCl - Attantional Conference	च्याताली (Jakobo <mark>0.0049</mark> वेदिक प्रतिकार सर्वीकारी	0.002 - 0.01
	Riboflavin	0.0003 0.003 5mg/ml	0.0001 7 0.0006
	Thiamine HCl	0.003	0.001 - 0.006
	HSA		•
20	Hyaluronate	0.1mg/ml	0.02 - 0.5

^{*} Concentrations are in millimoles unless otherwise indicated; the medium is aqueous.

Embryo Transfer Medium

The preferred embryo transfer medium contains the same formulation of

25 constituents as medium G2.2 except that a much higher concentration of hyaluronate is included. In the human reproductive system, research indicates that there is a same of receptor on the embryo for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and the hyaluronate and hyaluronate are hyaluronated and hyaluronate and hyaluronated and hyaluronate on the endometrium of the mother. Hyaluronate is thought to act like and biological glue that assists the embryo in binding to the endometrium and, 30 accordingly, supports implantation. The preferred amount and ranges of the accordingly supports implantation. constituents of the embryo transfer medium are depicted in Table 7.

Composition of Embryo Transfer Medium** 1777 - WORLA

ij.

 $\Delta spcraging$

N 151 (\$	Composition o	1 Dillot yo Transfer Iviou.	
Component		<u>A</u>	B
		Most Preferred	<u>Preferred</u>
en e		Concentration	· Range
· (1 = 1) . (1	,		internity
NaCi		90.08	19435 6 680.0 - 100

*(*1)

	See S. WO:00/32140		PCT/US99/28408
	KCl had forded very 12 3 A Carl E	१ द्याप्ति विदेशका ।	China and China
	NaH2PO4.2H2O		3.3 - 7.3
	Mg304./N20	1 .	1.5
	NaHCO3h na a mhi ko sega mibian a m	25	. 312 36 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
5	CaCl2.2H2O		15 - 30.0 0.8 - 2.8
		3.15	
	NaLactate (L-isomer)	5.87	0.5 - 5.5
	NaPyruvate	0.1	2.0 - 20.0
10	Alanine Section 1997	0.1	0.01 - 1.0 0.01 - 0.5
10		Ö.1	0.01 - 0.5
	Asparagine	0.1	0.01 - 0.5
72:	Glutamate	0.1	0.01 - 0.5
	Alanyl - Glutamine Glycine	1	0.01 - 0.5
15		0.1	0.01 - 0.5
13	Serine Serine	0.1	0.01 - 0.5
	L'-Arginine-HCl	0.1	0.01 - 0.5
	L-Cystine 2HCl	0.6	0.1 - 1.2
	L-Histidine-HCl-H2O	0.1	0.05 - 0.25
20	L-Isoleucine	0.2	0.1 - 0.4
	L-Leucine	0.4	0.1 - 0.8
	L-Lysine-HCl	0.4	0.1 - 0.8
	L-Methionine	0.4	0.1 - 0.8
	L-Phenylalanine	0.1	0.05 - 0.25
25	L-Threonine	0.2	17 - 0.1 - 0.4
	L-Tryptophan	0.4	0.1 - 0.8
	L-Tyrosine 2Na	0.5 0.2	0.1 - 0.9
	9.5 L-Waline	0.4	0.1 - 0.4
	D-Ca Pantothenate	0.002	0.1 - 0.8
30	Choline Chloride	0.002	0.001 - 0.004
	Folic Acid	0.0023	0.003 - 0.01
	i-Inositol	0.0111	0.001 - 0.0045
	Niacinamide St.	0.0082	0.005 - 0.02
25	Pyridoxal HCl	0.0049	0.004 - 0.016
35	Riboflavin	0.0003	0.002 - 0.01 0.0001 - 0.0006
	Thiamine HCl img ni	0.003	0.001 - 0.006
	Hyaluronate	0.25mg/ml	277770.05 ¹ -1.0
	Control supposed in and or success	Other 1908 in contin	अध्यक्षेत्रं अध्यक्षक विद्याले विदेश

*Concentrations are in millimoles, unless otherwise indicated; the medium is aqueous.

8. <u>Cryopreservation Medium</u>

40

The present invention involves a separate medium to be used in cryopreservation of the oocyte and embryo. The preferred formulation to be used includes ionic constituents and a buffer, preferably MOPS or HEPES, as well as the

carbohydrates lactate, pyruvate and glucose. Optionally, HSA may be included. In addition, the medium may include certain additives, glycerol, ethylene glycol, DMSO. propanedial, and/or sucrose. The preferred amounts and ranges of the constituents of the cryopreservation medium are depicted in Table 8. 40 (2)

10.0

1 37 Table 8 Composition of Cryopreservation Medium*

PROPERTY OF CONTRACTORS

305020

garage to

and multiple end

* .	-1 - €	- 5# 		The service and
• • .	Component		<u>A</u>	\mathbf{B}
4.5			Most Preferre	d Preferred Range
			Concentration	1
	NaCl		90.08	75.0 - 105
:	KCI		5.5	50 7.5 1914 Sunning 3.5 - 7.5
10	MgSO₄, 7H₂O		2	108 5 Mar 0.4 - 4
	N 000 / 01100	5 5 ·	*	Set tDR-earbirs (st
	Na2PO4.2H2O	1.5	0.25	0.17-1.5
		•	•	The safe factor of the safe state of the safe st
5,		.*		1999 seed with the seed of the
	NaHCO ₃	:	5	2.0 - 10
••-	MOPS/HEPES	, E	20	
 24	WOI S/IIEI ES	1.0	. 20	
1773 - 1772		•.		nudering of the
٠٠.	C CI AIL O		•	La Faccine 291e
	CaCl ₂ .2H ₂ O	É	I	Call 0.5 - 2.0
1	*f. **	83913		out minted to Co.
, 1		*****		DOD BOTH A SHIPPERSON
15	NaLactate (L-isomer)	Eliter Contract	5.87	2.0 20
•	NaPyruvate	11501	0.32	ichicale) Selmon <mark>0.1</mark> 411.0
	CASO C	St. 10 1	0.54	
	Glucose	POR DATE OF	1	10H 16.00 0.5 - 5.5
ϵ	HSA	1,000.0	F/ 1	E 19 S 150 K 15 T 1 T 1 T 1 T 1 T 1 T 1 T 1 T 1 T 1
8.4	6 HSA	£00.6	5mg/ml	1721 milita 01-10
•				

ADDITIVES

· .

20 Glycerol and/or ethylene glycol and/or DMSO and/or propanedial and/or sucrose Range for all except sucrose is 2 to 20%; range for sucrose is 0.1 to 1M complete the complete sucrose is 2.10 in the complete Concentrations are in millimeters unless otherwise indicated; the medium is aqueous 30000

O PISSUAL O

В. Sequential Culture Media Process

र है । इस विकास कार्युक्त कर जाता महिला विकास की कार्या कार्युक्त है Instead of immersing human reproductive cells in a single culture medium of a gallete was a mendigated.

and the state of the second of the

throughout the various procedures used in IVF, the present invention involves as the state of th 25

process by which the reproductive cells may be moved through a sequence of distinct culture media as the various IVF procedures are carried out. In one aspect of the invention, the culture media are specifically formulated to provide a physical environment similar to that found within the female reproductive tract and conducive to growth and development of human reproductive cells during various stages of the IVF process. In a further aspect of the invention, the specifically formulated culture media can be applied to support the reproductive cells in one or more of the following procedures: oocyte retrieval and handling; oocyte maturation; ordinary fertilization; oocyte, zygote and embryô examination and biopsy; embryonic development to the eight-cell stage; embryonic development to the blastocyst stage; embryo transfer; and cryopreservation. Most preferably, the media will be applied sequentially during each of the applicable stages of the IVF process to which the media have been adapted. It should be noted that there is significant variation among clinics and laboratories as to equipment and specific procedures used to accomplish each of the principal steps in the IVF process. The present invention contemplates that the sequential media and process described herein may be utilized and/or readily adapted for use with the wide variety of equipment and procedures employed in IVF practice. What follows is a more detailed discussion of exemplary applications of the media during IVF and related methodology. The property of the second .}

20 1. Oocyte Retrieval and Handling; Embryo Handling

น พิธา สมัย และสมาชิกเล เส่ มีของสมาชิสส และสามาชิก เรงดิดเด

5

10

15

25

30

Referring to Fig. 1, an initial procedure in the illustrated IVF process 100 is oocyte removal or retrieval (102) from the mother's ovary. This is typically performed vaginally using a fine needle attached to and guided by a transvaginal ultrasound probe. The needle is ordinarily connected to fine Teflon tubing and thence to an aspiration regulator controlled by a vacuum regulator. The aspirate is collected in test tubes or other appropriate vessels, containing medium. The medium may be used to preliminarily wash the needle and tubing, and other equipment used in the procedure. In some clinical settings, the medium may also be used with a specially adapted needle to flush the follicle and aid in removal of the oocyte. The medium, equipment used, and aspirate are maintained, so far as possible, at 37 degrees Celsius. If a bicarbonate buffer system is used in the medium, the procedure ordinarily is

atmosphere. In the absence of such atmospheric controls, the medium must contain a MOPS or HEPES buffering system.

The illustrated process 100 present invention contemplates that the oocyte retrieval and handling medium may be used in each phase of the retrieval process. The process of using the oocyte retrieval and handling medium may involve washing any equipment that may come into contact with the oocyte during removal from the ovary, and that may be used to aspirate, flush and/or wash the oocyte during the removal and collection process. Following removal from the ovary, the oocyte may be washed with medium. Optionally, the oocyte may be stored in the medium for a period.

In addition, it is contemplated that the medium may be used during other clinical or laboratory procedures where the oocyte is manipulated or handled, and also in procedures where the embryo is manipulated or handled, especially where these occur outside the isolette. Examples would include examination of the oocyte following retrieval from the mother, examination of the oocyte following the fertilization step, and examination of the embryo to determine whether it has developed the eight-cell stage. In each of these examples, the oocyte/embryo would be bathed in the medium as it is withdrawn by pipette from the culture dish or test tube, and would remain immersed in the medium while examined under a microscope or with other equipment. The illustrated implementation of the invention also contemplates that an alternative formulation of this medium, which is calcium and magnesium free, may be used during biopsy procedures.

performed vogmatly using a fine arealle after hear o and guid of the areasy that is a considered.

2. Occyte Maturation

if resolved probe. The aced of the crimerity connected to the first on arbitrary and the con-

5

10

15

20

25

30

In the event the collected oocytes are immature, the illustrated process 100 envisions that a second medium may be used to support and promote development of the oocyte during maturation (106). The oocyte maturation medium would ordinarily be used to treat and mature the oocyte following a collection procedure, in which the oocyte is retrieved from the ovary using oocyte retrieval and handling medium. The retrieval and handling medium and maturation medium have a very similar backbone of ionic constituents and amino acids and glutamine, such that as the oocyte is moved

from one medium to another it experiences minimal ionic shock. The illustrated process 100 includes immersing the oocyte and surrounding cumulus cells in the maturation medium for a period of about 30-48 hours, or until the oocyte is mature. The illustrated process 100 then contemplates removing the oocyte from the maturation medium and immersing it in either sperm preparation and fertilization medium or ICSI medium for purposes of fertilization.

In accordance with the invention, the oocyte maturation medium may be applied to the oocyte retrieval process (102), in place of the oocyte retrieval and handling medium described herein. Additionally, a conventional culture medium, such as Ham's F-10 or medium TCM-199 with or without a HEPES buffer, may be employed for immature oocyte retrieval and handling, before immersion of the oocyte in the maturation medium of the present invention. Once maturation is complete, the oocyte will be immersed in a medium for ordinary IVF fertilization procedure (110), or will be immersed in an ICSI medium in preparation for assisted insemination through an ICSI procedure (112).

3. Sperm Preparation and Fertilization

5

10

15

20

25

30

The illustrated process 100 contemplates that the collected oocytes will ordinarily be washed and immersed in, and allowed a period of pre-incubation culture within, a first portion of the sperm preparation and fertilization medium. This period of pre-incubation culture (iC4) may last up to about six (6) hours. Oocytes permitted a period of pre-incubation culture typically have higher fertilization rates.

The process 100 also contemplates that the sperm may be separately washed and stored in a second portion of the sperm preparation and fertilization medium to purge it of bacteria and any other contaminants that may be present. Sperm preparation (108) may involve dilution of semen with the medium, centrifugation, and resuspension of the concentrated sperm in a new portion of medium. In the "swim up" method of sperm preparation, the medium containing sperm is centrifuged, the medium is drained off, and a new portion of medium is poured over the spundown sperm pellet. The sperm is given a period to "swim up" into the fresh medium. That layer of fresh medium, containing the more motile sperm, is then poured off and centrifuged, and the process is repeated. In another aspect of the invention, the sperm

preparation and fertilization medium may be used in one or more gradient separation procedures, such as the Percoll procedure. The present invention envisions that the sperm preparation and fertilization medium may be used as the medium in any of the sperm preparation procedures that may be used for IVF. 30.001

5

10

15

20

25

30

Once the sperm is prepared (108), the sperm is then examined and counted while in medium, and a desired quantity is added to the portion of medium which contains the oocyte. The sperm and oocyte are permitted to remain together in the medium for a period of up to several hours, and, in some laboratories, for a much longer period, as long as about sixteen (16) to eighteen (18) hours. The invention further contemplates that, following a period of immersion in the medium with sperm, the oocytes will be removed and examined (114) to determine whether fertilization (110) has occurred. When removed for examination, the oocytes will continue to be bathed in the sperm preparation and fertilization medium if the examination can be conducted in an isolette. If not, then, as noted above, the oocyte retrieval and handling medium may be used for handling and examination of the oocytes.

. 1

15

25

30

4. Fertilization by Direct Injection of Sperm into the Oocyte (ICSI & Technique)

In the ICSI process (112), sperm may be directly injected into the cytoplasm of the oocyte through a very fine pipette or needle. The process 100 contemplates washing the sperm with a portion of the ICSI medium containing hyaluronate and/or PVP, and then placing the sperm in the medium. The process 100 further involves drawing a microvolume of the medium containing sperm into the pipette and then injecting the medium and sperm into the interior of the oocyte as becase and barete had

The illustrated process 100 further contemplates that the obcyte may be bathed in another portion of the ICSI medium during the ICSI process. An alternative design of the ICSI medium may be used, supplemented with hyaluronidase, for denuding pretreatment (105) of the oocyte prior to the ICSI process. Pretreatment involves immersing the oocyte in the ICSI medium supplemented with hyaluronidase for a period until the oocyte becomes denuded of all or most of its surrounding cumulus cells. Following pretreatment, the oocyte is injected with sperm carried in a separate portion of medium, using an ICSI pipette, as provided above.

1.6 表示 医鹿克曼

After the ICSI injection process (112) is complete, it may be necessary to examine (114) the oocyte to evaluate whether fertilization has been effective and the oocyte is intact and healthy. Examination may occur in the ICSI medium bathing the oocyte, or may occur in the oocyte retrieval and handling medium as described above.

5. Embryonic Development to Eight-Cell Stage

Les en blue des cell ettes c

5

10

Medium G1.2 is applied to the early embryo, following formation of the zygote. After the zygote is identified, it is washed with medium G1.2, and then immersed in G1.2 medium for a culturing period (116) of up to about forty-eight hours. During this time the embryo undergoes development from the one-cell to around the eight-cell stage, and is removed at about the eight-cell stage. Examination (118) of the embryo may occur in the G1.2 medium, or in the oocyte retrieval and handling medium, as described above.

6. Embryonic Development to Blastocyst Stage

The illustrated process 100 contemplates that medium G2.2 will be used to

15 culture (120) the developing embryo to the blastocyst stage, preferably from about the
eight-cell stage to about the one-hundred-cell stage. The process 100 also
contemplates that, once the embryo reaches the blastocyst stage, and assuming that
the embryo is judged on examination (124) to be viable, it is removed from the G2.2
medium and prepared for transfer into the uterus. In some laboratories, the G2.2

20 medium may, optionally, be used for embryo transfer as well. Examination (124) of
the embryo may occur in the G2.2 medium or in the oocyte retrieval and handling
medium, as described above.

7. <u>Embryo Transfer</u>

The process 100 contemplates that the embryo transfer medium will serve as a carrier for the embryo as it is transferred (126) back into the mother. The embryo will be bathed in the transfer medium, the medium containing the embryo will be drawn into the transfer catheter, the catheter will be inserted into the mother's uterus, guided by an ultrasound probe, and the medium containing the embryo will be injected into the uterus.

WO 00/32140

5

- PCT/US99/28408

8. Cryopreservation of (St.) serves to cost of \$250 years 14.

The cryopreservation medium may be used for storing, freezing, thawing, witrification, and warming the oocyte, prior to fertilization. The same medium may be used for storing, freezing, thawing, vitrification, and warming the cleavage stage embryo, as well as the embryo in the eight to one hundred cell stage.

123 B. Day

While the present invention has been described in relation to one embodiment, it will be appreciated that the invention may be utilized in numerous additional embodiments and procedures. Such additional embodiments and procedures are within the scope of the present invention, as defined by the claims which follow.

amin of the etcheloof origin and is removed at the origin of the sign of the sign of the continue of the conti

and the second of the second o

Harris of the second of the se

As the effective and proceed to the control of the

Hu-on Ironsist

35

Charles for an expected for the five state of the expect of the control of the co

5

10

15

20

25

ARTHUR AND

What is claimed is: 100 february on the first and in the second of the s

A method for use in an IVF process, wherein the process involves some or all of the stages of: oocyte retrieval and handling; oocyte maturation; sperm preparation; fertilization; oocyte, zygote and embryo examination and biopsy; embryo development; embryo transfer; and cryopreservation said method comprising the steps of:

supporting reproductive cells in a first support medium during a first stage of said stages, said first support medium including a core group of salts; and

supporting reproductive cells in a second support medium different than said first support medium during a second stage of said stages, said second support medium including substantially said same core group of saits as said first support medium, said core group of salts utilized in both of said first and second support media thereby minimizing any stress and trauma to reproductive cells incident to transfer between the first and second support media;

wherein no more than one of said first and second stages is one of said embryo development stage and said embryo transfer stage.

- 2. A method as set forth in Claim 1, wherein said first stage is one of embryo examination and oocyte retrieval and handling.
- 3. A method as set forth in Claim 2, wherein said first support medium comprises water, ionic constituents and a buffer.
- Amothed as set forth in Claim 2, wherein said first support medium comprises one of 4-Morpholinepropanesulfonic acid (MOPS), N-2-hydroxyethylpipgrazine Nil 2-ethane sulphonic acid (HEPES) or bicarbonate.
- 5. A method as set forth in Claim 2, wherein said first support medium compasses carbohydrates; be a month of the control of t
- 6. A method as ser forth in Claim 2, wherein said first support medium comprises non-essential amino acids:
- 7. A method as set forth in Claim 2, wherein said first support medium comprises glutamine and size should be a selected to the support medium.
- 8. A method as set forth in Claim 2, wherein said first support medium comprises antibiotics.

9. A method as set forth in Claim 1, wherein said first support medium is free from calcium and magnesium and said first support medium is used in biopsy procedures.

10. A method as set forth in Claim 1, wherein said first stage comprises to cocyte maturation.

5

10

15

20

25

30

- 11. A method as set forth in Claim 10, wherein said step of supporting reproductive cells in a first support medium comprises supporting an oocyte in said first support medium for a time period following oocyte collection to promote gate 1 and development prior to fertilization.
- 12. A method as set forth in Claim 10, wherein said first support medium is comprises magnesium and calcium disbursed in an aqueous solution.

- 14. A method as set forth in Claim 10, wherein said first support medium comprises one or more growth factors such as insulin transferin selenium (ITS); one with insulin-like growth factor (IGF), and epidermal growth factor (EGF).
- 15. A method as set forth in Claim 10, wherein said first support medium force comprises one or more hormones such as follicle stimulating hormone (ESH) and human chorionic gonadotrophin (hCG).
- 16. A method as set forth in Claim 1, wherein said first stage comprises one of sperm preparation and fertilization. The Management of the
- 17. A method as set forth in Claim 16, wherein said first support medium to comprises carbohydrates.
- 18. A method as set forth in Claim 16, wherein said first support mediting comprises one or more of bicarbonate, glutathione, HSA and hypersonate.
- 19. A method as set forth in Claim 16, wherein said first support medium # 60 comprises antibiotics.
- 20. A method as set forth in Claim 16, wherein said first support medium (20) comprises nonessential amino acids.
- 21. A method as set forth in Claim 16, wherein said first support medium with is free of EDTA.

22. 20 A method as set forth in Claim 1, wherein said first stage comprises oocyte retrieval and handling and said second stage comprises one of sperm preparation and fertilization.

23. A method as set forth in Claim 22, wherein said second support medium has an elevated concentration of sodium as compared to said first support medium.

5

10

15

20

25

- 24. A method as set forth in Claim 22, wherein said second support medium has an elevated concentration of phosphate as compared to said first support medium.
- 25. A method as set forth in Claim 1, wherein said first stage utilizing said first support medium is part of a process of intracytoplasmic sperm injection (ICSI).
 - 26. A method as set forth in Claim 25, wherein said ICSI process comprises removing cumulus cells from an oocyte, incubating sperm, and injecting the sperm into said oocyte; and
- into said second
 support medium:
 - 27. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process is free from phosphate.
- 28. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises one of MOPS process, HEPES and bicarbonate.
 - used in said ICSI process comprises carbohydrates.
 - used in said ICSI process is free of glucose.
 - used in said ICSI process comprises non-essential amino acids.
 - 32. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises glutamine.

1.24

30 A method as set forth in Claim 26, wherein said first support medium is used for supporting said sperm as part of said ICSI process and comprises one of hyaluronate or polyvinylpyrolidorie (PVP).

WO 00/32140 PCT/US99/28408

34. A method as set forth in Claim 25, wherein said first support medium comprises magnesium and calcium in an aqueous solution. He is to be a like to be the set of the comprise magnesium and calcium in an aqueous solution.

- 35. A method as set forth in Claim 25, wherein said first stage comprises denuding an oocyte and said first support medium comprises hyaluronidase.
- 36. A method as set forth in Claim 1, wherein said first stage comprises to embryonic development.

5

10

15

20

25

- 37. A method as set forth in Claim 36, wherein said step of supporting reproductive cells in a first support medium comprises supporting a zygote in said first support medium for a time period that is one of at least 48 hours or through at least the eight-cell stage.
- 38. A method as set forth in Claim 36, wherein said first support medium? comprises carbohydrates.

0:

- 39. A method as set forth in Claim 36, wherein said first support medium of comprises non-essential amino acids.
- 40. A method as set forth in Claim 36, wherein said first support medium comprises one or more of HSA, and hyaluronate.
- 41. A method as set forth in Claim 36, wherein said first support medium comprises glutamine.
- 42. A method as set forth in Claim 41, wherein said glutamine comprises and alanyl-glutamine.
- 43. A method as set forth in Claim 1, further comprising the step of supporting reproductive cells in a third support medium different than said first and second support mediums during a third stage of said stages on 2000 of 1200 bits of the second support mediums during a third stage of said stages on 2000 of 1200 bits of the second support mediums during a third stage of said stages.
- 44. A method as set forth in Claim 43, wherein both said second stage and said third stage comprise embryo development and transfer. at 1250 to 120 t
- 45. A method as set forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has a depressed concentration of one of lactate and pyruvate relative to said second medium.
- 30
 46. A method as set forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has:

 an elevated concentration of glucose relative to said second support medium.

WO 00/32140 PCT/US99/28408

47. A method as set forth in Claim 36, wherein said step of supporting reproductive cells in a first support medium comprises supporting an embryo in said first support medium for a time period that is one of from about 48 to 96 hours and from about the eight-cell stage to about the one hundred cell stage.

48. A method as set forth in Claim 36, wherein said first support medium comprises non-essential amino acids and is free from taurine.

5

- 49. A method as set forth in Claim 36, wherein said first support medium comprises essential amino acids.
- 50. A method as set forth in Claim 36, wherein said first support medium 10 comprises vitamins.
 - 51. A method as set forth in Claim 36, wherein said first support medium comprises HSA.
 - 52. A method as set forth in Claim 36, wherein said first support medium is free from EDTA.
- 15 53. A method as set forth in Claim 36, wherein hyaluronate is added to said first support medium for embryo transfer.
 - 54. A method as set forth in Claim 1, wherein said first stage comprises cryopreservation.
- 55. A method as set forth in Claim 54, wherein said first support medium 20 comprises one of MOPS or HEPES.
 - 56. A method as set forth in Claim 54, wherein said first support medium comprises carbohydrates:
 - 57. A method as set forth in Claim 54, wherein said first support medium comprises HSA.
- 25 58. A method as set forth in Claim 54, wherein said first support medium comprises one or more of glycerol, ethylene glycol, DMSO, propanediol and sucrose.
 - 59. A method as set forth in Claim 36, wherein said first support medium comprises EDTA.
- 60. A method as set forth in Claim 54, wherein said first support medium comprises nonessential amino acids.

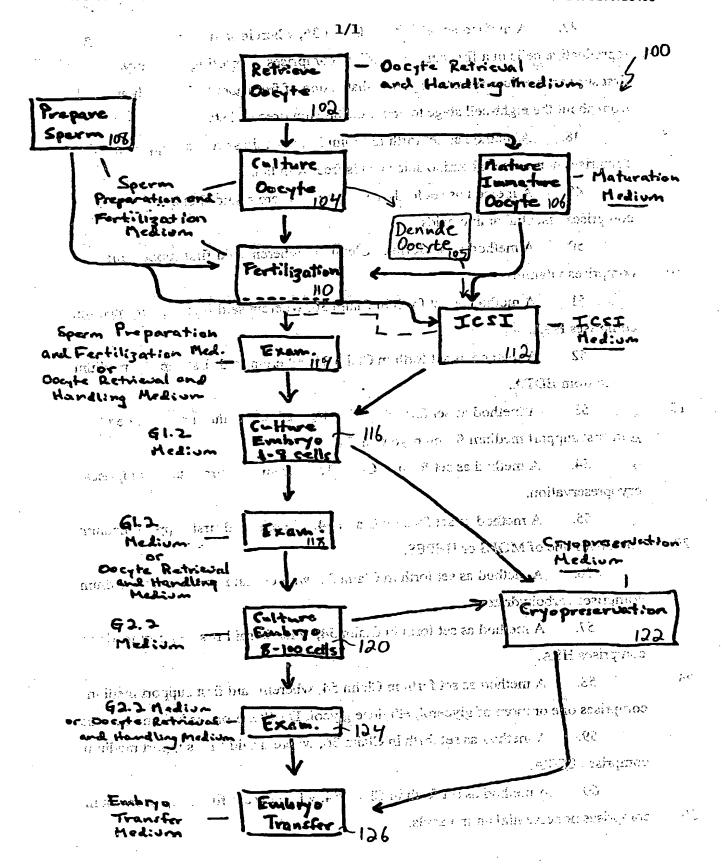


Figure 1

.

INTERNATIONAL SEARCH REPORT

International application No. PCT/US99/28408

A. CLASSIFICATION OF SUBJECT MATTER	" 是自由、政策工 (1)。 计 (4) 4	
IPC(7) :A61F 5/58; A01N 1/02	and the majorate of the control of t	
00 00 .455/2, 000/25, 24, 25	ကြောင်းသည်။ ကြောင်းသည်။ ကြောင်းသည်။ မော	e u de fe
According to International Patent Classification (IPC) or to both	national classification and IPC	<u>-</u>
B. FIELDS SEARCHED		<u> </u>
Minimum documentation searched (classification system follow	And the second s	
. •	ed by classification symbols)	
U.S. : 435/2; 600/23, 24, 25		: .
Documentation searched other than minimum documentation to the	he extent that such documents are included	in the fields searched
·	- .	·
		3
	<u> </u>	128 - Maria 4
Electronic data base consulted during the international search (r	name of data base and, where practicable	search terms used)
Medline, Biosis, US Pats on WEST		,
	•	
		-
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category. 1 to Citation of document, with indication; where a	peropriate of the relevant passages	Relevant to claim No.
		Trois vant as chain 110.
X ABEYDEERA et al. Fertilization and	Subsequent Development In	1-3 · · · · · · · · · · ·
Vitro of Pig Oocytes Inseminated	in to Modified The Description	1-3
Madical Part of the Control of the C	in a Modified Tris-Buffered	Ratio Searcher of the
Medium with Frozen-Thawed Ejacula	ated Spermatozoa. Biology of	
Reproduction. 1997, Vol. 57, pages	729-734, see Materials and	er e e
Methods.	•	
	·	<u> </u>
		•
		; 3
	1	ŧ
		ţ
	·	:
		•
·	•	
 त्यां के कार्याच्या कानुस्थान मध्या चर्च है। त्या बहारण प्राप्त अति एक प्रदेश के त्यां के त्यां के 	and free and the state of the same	
		· · · · · · · · · · · · · · · · · · ·
a con finality in a collection for which we have the analysis of consuming and		
	rosa (ku 📑	Le qui te
an trough day an fan raam weel plat ste abilit en eet hou being glade horse	and the same of th	
and the training and a management of the contract of the contract of	No. 7 Paris Control of the Control o	THE PRINTS HAVE BEEN A
Further documents are listed in the continuation of Box C		
	See patent family annex.	
Special categories of cited documents:	"T" later document published after the inte	
'A' document defining the general state of the art which is not considered	date and not in conflict with the appli the principle or theory underlying the	cation but cited to understand
to be of particular relevance	•	
'E' carlier document published on or after the international filing date	"X" document of particular relevance; the considered novel or cannot be consider	claimed invention cannot be ed to involve an inventive step
L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other	when the document is taken alone	! *
	document of particular relevance; the	
O document referring to an oral disclosure, use, exhibition or other:		
means	being obvious to a person skilled in the	
P° document published prior to the international filing date but later than the priority date claimed	*A. document member of the same patent	family
Date of the actual completion of the international search	Date of mailing of the international sear	ch report
06 APRIL 2000 1 4 4 7 18 18 18 18 18 18 18 18 18 18 18 18 18	18 APR 2000	1
		,
Name and mailing address of the ISA/US	Authorized officer	
Commissioner of Palents and Trademarks) in	上
Box PCT Washington, D.C. 20231	SANDRA SAUCIER	ν
Facsimile No. (703) 305-3230	ع بدراد و	r
	Telephone No. (703) 308-0196	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/28408

Sox I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)	en de la companya de La companya de la co
his international report has not been established in respect of certain claims under Article 17(2)(a) for the following reason	(15 5) s; ~ g() 1
Claims-Nos.: Claims-Nos.: because they relate to subject matter not required to be searched by this Authority, namely: 2. An about	48 34
12 12 12 12 12 12 12 12 12 12 12 12 12 1	,
ය. දෙන්ව දෙන නම් වැනිව දෙන නම් සහ සම්බන්ත කොට සහ නම් වන කොට සහ නම් මෙන් වැනිව සිට සම්බන්ත විය වෙන සම්බන්ත	
Claims Nos.:	
because they relate to parts of the international application that do not comply with the prescribed requirement an extent that no meaningful international search can be carried out; specifically real to have a record or	nts to such
TEST of a second of the company of t	Sello Ma
TE STORE THE TEST OF THE TEST STORE AND A STORE AND A STORE AND A STORE AS A	inina da
. Claims Nos.:	
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rufe	€(δ.4(a):
x II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)	X
is International Searching Authority found multiple inventions in this international application, as follows:	
Please See Extra Sheet. 46 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	4 .
- Androeu	
As all required additional search fees were timely paid by the applicant, this international search report covers	all search
claims.	, 4,, 54_,4,,
As all searchable claims could be searched without effort justifying an additional fee, this Authority did not of any additional fee.	invite payn
As only some of the required additional search fees were timely paid by the applicant, this international search	h report co
only those claims for which fees were paid, specifically claims Nos.	
egans along the control of the Contr	ئىرە بە ئەنىسا، بىلىسا،
and the first of control of the cont	٠,٠
in a complete control of the control	
Assument world? A say there is the problem of an interpretable of a say the same of the sa	. 1.
No required additional search fees were timely paid by the applicant. Consequently, this international search fees were timely paid by the applicant. Consequently, this international search fees were timely paid by the applicant.	earch repor
1-3 Frank as the part of the graph of the gr	
by a section of the control of the c	
The graph of the second of the particle of the second of t	
nark on Protest The additional search fees were accompanied by the applicant's protest.	Carate
No protest accompanied the payment of additional search fees.	Name teet
	ansin 40 1014 m 13 1000 mm
ACTION (CONT.)	1.12-4.

Ì

Franklik Lindovinedakti (k. 1927) Franklik Girlandovinedakti (k. 1927)

INTERNATIONAL SEARCH REPORT

建设产业公司将自由企

International application No. PCT/US99/28408

10 35 West 191

and a second

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

IN THE HALL SEE ARE LIKE LITTLE.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

First, each stage claimed in the method is a distinct species, such as the species of the stage of claim 2, the species of the stage of claim 10, of claim 22, of claim 25, of claim 36, of claim 43, and the stage of claim 54.

Further, each medium is a distinct species. For example, the species of the stage of claim 2 has distinct species of media from one of claims 3-9 (7 species). The species of the stage of claim 10 has the media of the species of claims 12-15 (4 species). The species of the stage of claim 16 has the species of the media of claims 17-21 (5 species). The species of the stage of claim 22 has the media of the species of claims 23 and 24 (2 species). The species of the stage of claim 36 has the media of the species of claim 36 has the media of the species of claims 38-42 (5 species). The species of the stage of claim 43 has the media species of claims 45 and 46 (2 species). The species of the stage of claim 54 has the media of the species of claims 55-60 (6 species). This is a total of 40 distinct species of stages of claim 1 and the media of the dependent claims.

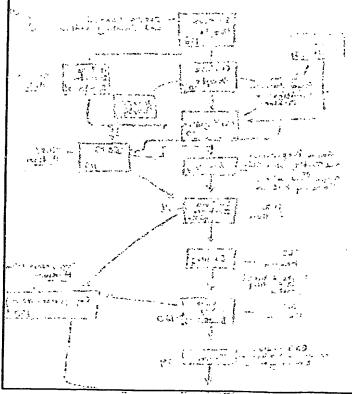
The following claim is generic: claim 1.

. Printing of the

58.3

2.6

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: The stages lack a special technical feature because all of the stages of the IVF process are known in the art. The media used in the method lack a special technical feature because it is well known in the art to modify the media while retaining at least two of the same salts, which fulfills the limitation recited as "a core group" between stages of an IVF procedure. For example, porcine occyte-cumulus complexes were incubated in NCSU medium containing FF, then incubated in NCSU medium without FF, then the fertilized occytes were incubated in NCSU medium with BSA as taught by Abeydeera et al. NCSU is used as the base medium and would have the same "core salts" throughout the process. The process as claimed lacks a special technical feature, and therefore, lacks unity of invention.



The sold tending of realization as those and the sold tending of realizations as the sold tending of realizations as the sold tending of tending of the sold tending o

Form PCT/ISA/210 (extra sheet) (July 1998)* .

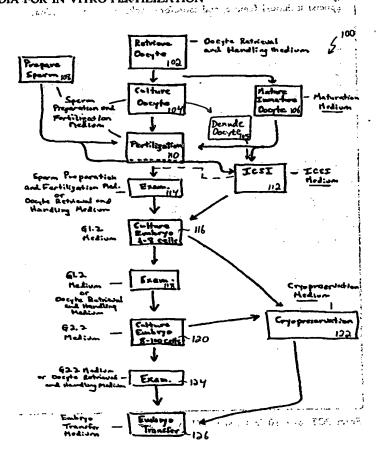


INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT, COOPERATION TREATY (PCT)

(51) International Patent Classification 7:	(11) Internati nal Publication Number: WO 00/32140
A61F 5/58, A01N 1/02	(43) Internati nal Publication Date: 8 June 2000 (08.06.00)
(21) International Application Number: PCT/US99/28- (22) International Filing Date: 30 November 1999 (30.11. (30) Priority Data: 09/201,594 30 November 1998 (30.11.98) (71) Applicant: IVF SCIENCES COLORADO, INC. (US/L Suite 300, 799 E. Hampden Avenue, Englewood, CO 80 (US). (72) Inventors: GARDNER, David, K.; 9927 Clyde Circle, Hi lands Ranch, CO 80126 (US). LANE, Michelle; 1661 W	(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(74) Agents: FISCHMANN, Kent, A. et al.; Holme Roberts & Ov LLP, Suite 4100, 1700 Lincoln Street, Denver, CO 80 (US).	ven
 Control x 2 (el per production) までま (機能の) 100 cm A Marcha attentable (terrel) (a APM) 12 (el per production) (a APM) (a APM)	gram and a typical or of a set of to observed to strong and a set of the set

(57) Abstract

Instead of immersing human reproductive cells in a single culture medium throughout the various procedures used in IVF, a process is provided by which the reproductive cells may be moved through a sequence of distinct culture media as the various IVF procedures are carried out. In one implementation, the culture media specifically formulated to provide a physical environment similar to that found within the female reproductive tract and conducive to growth and development of human reproductive cells during the various stages of the IVF process. In this regard, specifically formulated culture media can be applied to support the reproductive cells in one or more of the following procedures: oocyte retrieval and handling; oocyte maturation; ordinary fertilization; oocyte, zygote and embryo examination and biopsy; embryonic development to the eight-cell stage; embryonic development to the blastocyst stage; embryo transfer; and cryopreservation.



S. 61 CONTINA

16 Destriction of the American Standard wife in Annual Commence of the Annual Commence of the

emiliar throat was seen about to a thirty and critical application राष्ट्रभावता के प्रतिकार के प्रतिकार के प्रतिकार के प्रतिकार के प्रतिकार के लिए के प्रतिकार के प्रतिकार के प्र เมืองการ พระเสดิ จังเลก กระการเกาะสังได้กระเวลาการเลกสุด เกาะการเลก เลก 57 for town with pales the continue they are they are

ริยาสุด พ.ศ. และว่า เรียก และใหญ่สิทธิสุด โดยโดยที่ ค.ศ. ค.ศ. และ และสามารถ และ เกล

Like experies within a case to transpagned from the cities of the configuration by the configuration. to subject to applied a comment to the congress best for expression to the region of the contract of the contr workling and its compagn to a little property of the second of the 13 73 13 was stored and in the cool allocations, or become that took black in the cool of the cool and the In the Commence of the commenc

PRINTED A PER EXPANDED CART CART CENTER OF EARLY AND BUT The second of the second of the second of OZ.

2000年前からのとは、北直山地をおける。。 こうかい かいしんかい A Committee of the contract of

marks a compared with him a copy of the score of the score

authors bouter and the authorized the FOR THE PURPOSES OF INFORMATION ONLY ्र कर प्रवृक्त करते प्रकार स्वीत है के प्रवृक्त कर है। Maria A

		Codes used to identify:	States pai	rty to the PCT on the front	nages of	f pamphlets publishing inte		1:4:
	:	为 (75% GA) 對議	୍ଟ୍ରେମ୍ବର	llusem you garder hig.	P-Boo o.	bence brouging inte	mationa	applications under the
AL		Albania	ES				•	
AM		Armenia 10 10 (1997)		Spain	LS	Lesotho	SI	Slovenia
AT	1.	Austria			CAT:	Lithuania	SK	Slovakia
			FR	France `	LU	Luxembourg	SN	Senegal
AU	٠	Australia Azerbaijan	GA.	Gaboo To To Tall Control	LV	Latvia of Production of	SZ.	Swaziland
AZ			`GB	United Kingdom	MC	Мовасо	TD	Chad
BA		Bosnia and Herzegovina	GE	Georgia	MD .	Republic of Moldova	TG	
BB		Barbados	GH	Ghana	MG	Madagascar		Togo
BE		Belgium	GN -	Guinea	MK		TJ	Tajikistan
BF	.53	Burkina Paso Graph 127			IVI P.	The former Yugoslav	TM	Turkmenistan
BG		Bulgaria	HU	Hungary	el Baralia	Republic of Macedonia	TR ···	Turkey
BJ		Benin	IE		ML	Mali -	TT	Trinidad and Tobago
BR		Brazil			, MN	- Mongolia	UA	Ukraine
BY		Belarus	IL.	ISIZEI	MR	Mauritania	UG	Uganda
	٠.,		IS	loeland	MW	Malawi	US	United States of America
				The say and the said of	MX	Mexico	UZ '	Uzbekistan
CF		Central African Republic	JP ·	Japan	NE	Niger	VN	Viet Nam
CG		Congo	KB	Kenya	NL :			
CH		Switzerland	KG	Kyrgyzstan	NO	Norway	ΥU	Yugoslavia
CI		Côte d'Ivoire	C KP	Democratic People's		.,	zw	Zimbabwe
CM	•	Cameroon	1 35	Republic of Korea	NZ			•
CN		China	KR	Republic of Korea	PL	Poland		•
CU		Cuba	-	-	PT 30	Portugal		
CZ		Czech Republic	KZ	Kazakstan	RO ·	Romania		
DE			LC	Saint Lucia	RU	Russian Federation		
		Germany	u	Liechtenstein	SD	Sudan		
DK		Denmark	LK	Sri Lanka	SE	Sweden		
EE		Estonia	LR	Liberia	SC	Singenore		

5

10

15

20

25

43 m

m~5 . .. *5

3 2513 11 - ta le interior defetti

 $\operatorname{cm} \operatorname{for}^* A$

 $\pi_{\mathcal{H}}(C_{i_1}, \mathcal{L}_{i_2})$

- 1 1 - 70°

1764 " 155

r

Pers 31

. 7

versa.

100

AMENDED CLAIMS

[received by the International Bureau on 19 June 2000 (19.06.00); original claims 1, 36 and 40 amended; new claims 61-127 added; remaining claims unchanged (17 pages)]

A method for use in a human IVF process, wherein the process involves some l. or all of the stages of: occyte retrieval and handling; occyte maturation; sperm preparation; fertilization; oocyte, zygote and embryo examination and biopsy; embryo development; embryo transfer; and cryopreservation said method comprising the steps of:

supporting human reproductive cells in a first support medium during a first stage of said stages, said first support medium including a core group of salts comprising at least two different salts; and

supporting human reproductive cells in a second support medium different than said first support medium during a second stage of said stages, said second support medium including substantially said same core group of salts as said first support medium, said core group of salts utilized in both of said first and second support media thereby minimizing any stress and trauma to human reproductive cells incident to transfer between the first and second support media;

wherein no more than one of said first and second stages is one of said embryo development stage and said embryo transfer stage.

- A method as set forth in Claim 1, wherein said first stage is one of embryo 2. examination and oocyte retrieval and handling.
- A method as set forth in Claim 2, wherein said first support medium 3. comprises water, ionic constituents and a buffer.
- A method as set forth in Claim 2, wherein said first support medium 4. comprises one of 4-Morpholinepropanesulfonic acid (MOPS), N-2-នៅ 🕠 សម្រាក់ស្រី ក្រុ ស្តែម្ 🗷 hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) or bicarbonate!
 - A method as set forth in Claim 2, wherein said first support medium 4. 10 5. N ar, vokrezaří řek ', ") comprises carbohydrates. . unr. a.P
 - A method as set forth in Claim 2, wherein said first support medium 6. gr comprises non-essential amino acids.
 - A method as set forth in Claim 2, wherein said first support medium alluance consists for all card. comprises glutamine. 1 Step 1 5 1.5 h17.

* :::

A method as set forth in Claim 2, wherein said first support medium 8. 30 his .. • • So Silvery & comprises antibiotics. .: 5 fi trans attions as 😂 ac defini post of section $\{i,j\}$

€.1

Acres 6

10

- A method as set forth in Claim 25, wherein said first support medium 34. comprises magnesium and calcium in an aqueous solution.
- A method as set forth in Claim 25, wherein said first stage comprises denuding an oocyte and said first support medium comprises hyaluronidase.
- A method as set forth in Claim 1, wherein said first stage comprises has become a star embryo andevelopment a company
 - A method as set forth in Claim 36, wherein said step of supporting reproductive cells in a first support medium comprises supporting a zygote in said first support medium for a time period that is one of at least 48 hours or through at least the eight-cell stage.
 - 38. A method as set forth in Claim 36, wherein said first support medium comprises carbohydrates.
- A method as set forth in Claim 36, wherein said first support medium comprises non-essential amino acids.
 - 15 40. A method as set forth in Claim 36, wherein said first support medium but the set with the set of the se
- A method as set forth in Claim 36, wherein said first support medium To surely sometimes, comprises glutamine. mi + 2 60 + 100
- 42. A method as set forth in Claim 41, wherein said glutamine comprises

 (1.34, 4.35) part (0.34-6.2 again to an O.3) (3.45)

 20 alanyl-glutamine
 - A method as set forth in Claim 1, further comprising the step of 43. is a contribution of supporting reproductive cells in a third support medium different than said first and second support mediums during a third stage of said stages.
- A method as set forth in Claim 43, wherein both said second stage and 44. 25 said third stage comprise embryo development and transfer.
- A method as set forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has south editif actions in the first of the agent of the second of the first and pyruvate relative to said second value of an orillomedium di oponi och el enlovig i i i i i i

ou, of discourse in the

30 A method as set forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has an elevated concentration of glucose relative to said second support medium.

 C_{i}

in the many there is the property of the property of the following the first of the An aqueous composition, comprising the components:

Control of the Contro

the first the thought the first at the latter A.

The area of the games of the

ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate, and The worlding in water:

(2) 完全200 (2013) (2013) (2013)

, क्या केंद्र वोष्ट्राय ठाउँ का भी

of a country of the condition of the condition of the period of a buffer to maintain the pH of said composition within the human physiological

range:

2. Q. 3.13 * Q.4.

the carbohydrates glucose, lactate and pyruvate; and ile organistical in application

alanine, asparate, asparagine, glutamate, alanyl-glutamine, glycine, proline, serine and

controlled may else in a action and is-

taurine.

o e i i gadi son ni bi ikini. The aqueous composition of claim 61, wherein said components are in the ARDO I SI MEDINE TE THE ROPERS

form and concentration in millimole per liter, unless otherwise noted, as follows: 4.

NaCl in the range 75-105; KCl in the range 3.5-7.5; NaH₂PO₄.2H₂O in the range of

.05-1.5; MgSO₄.7H₂0 in the range 0.2-4.0; NaHCO₃ in the range 2.0-10.0; and CaCl₂.2H₂O រត្តមានិស្ស ខេត្តិស្នឹង-ស្នែកស្នា

 \sim , in the range 0.8-2.8;

15 the buffer 4-morpholinepropanesulphonic acid (MOPS) with a concentration in the stor at support or what support and the modes in total range of 10.0-25.0;

glucose in the range .05-5.0, NaLactate (Lisomer) in the range 5.0-20.0. and

(NaPyruvate in the range 0.10-1.0, and have at both som A

A method as the forth is Officially specification of the man

trainem . Angean tribit has then normalis surges. Bryonal his of hear parties been of alanine in the range .01-0.5; asparate in the range .01 to 0.5; asparagine in the range .02 to 0.5; asparagine in the range .03 to 0.5; asparagine in the range .03 to the surgestion is the surgestion of the surgestion in the range .03 to the surgestion is the surgestion of the surgestion in the surgestion of the surgestion is the surgestion of the surgestion of the surgestion is the surgestion of the surgesti

20 0.01-0.5; glutamate in the range .01-0.5; glycine in the range .01-0.5; proline in the range

01-0.5; serine in the range .01-0.5 and taurine in the range .01-10.0, and alanyl-glutamine

ordine grability in the compact through the compactive to exin the range .01-2.0. 68.

5

- The composition of claim 62; wherein said buffer is N-2-63. 13.2 C 1 0 hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) and has a concentration in the range of 10.0-25.0
- The composition of claim 61, wherein said components do not include 64. calcium or magnesium
 - The composition of claim 61, further comprising an antibiotic. 65.
 - The composition of claim 61, further comprising at least one human 66. reproductive cell-selected from a group consisting of human gametes, human zygotes and 600 EA human embryos.
- 10 ...67. H. The composition of claim 61, wherein such composition is at least partially contained in a rigid housing.
- An aqueous composition, comprising the components: single description and in its constituents sodium, potassium, phosphate, magnesium, bicarbonate and on como <mark>calcium, in water;</mark> comedia a como a se como de la comedia de l
- 11.15 THE IS TO BE a buffer to maintain the pH of said composition within the human physiological range; or a wide on a contract

a.n. office mathe carbohydrates glucose, lactate and pyruvate; and sale in

alanine, asparate, asparagine, glutamate, glycine, proline, serine, and taurine

569 The composition of claim 68, wherein the form and concentration range, in

millimole per liter unless otherwise noted, of the components, are as follows: 20

ANDWELD AS INCOME ROLL COMENSACTOR OF THE PORT AND TO AN 75-100;

NaH₂PO4.2H2O 0.05 - 1.5;

A Company of the second	~ 1		ès
	NaLactate (1-isom	0.5 - 5.6; (er) 10 - 2:0 - 20; (h.h.) 0.1 - 0.5:	:वक्षाक्ष्मी हैं।
		0.1 - 0.5;	, .
5	NaHCO3	15-30, ^{6 3,6 3,6}	1 ID 62 W
to version and the state of th	S. CaCl2.2H2O	c seeds 0.892.8	4- <u>3</u>
	Alanine	0.01 = 0.5; (1.87)	policy, folia
	Asparate	0.01 - 0.5;	
and the second s	Asparagine 💛 😅	0.01 - 0.5; 0.01-0.5;	.ċ.€
10	Glutamate	0.01 - 0.5:	
on the second	- Glycine 100 miles	45 (0.61% 0.01% 0.55)	
	Proline	0.01 - 0.5:	
المعاودة المعارضة	Serine Service not sur-	ors a 0.01 0.5; and 34	itootayqali
Company of the Compan	launne	0.01 - 10.0.	•
		oryes.	eta decenti.

- 15 70, 100 70, 100 The composition of claim 68, comprising also glutathione.
 - 71. The composition of claim 69, comprising also glutathione in the concentration range, in milligrams per milliliter, of 0.5-5.0; and a contract the
- 72. The composition of claim 68, comprising at least one human reproductive cell selected from a group consisting of human gametes, human zygotes and human embryos.
- 20 73. The composition of claim 68, wherein such composition is at least partially contained in a rigid housing.
 - The composition of claim 68, comprising also human serum albumin. 74.
 - 75. The composition of claim 68, comprising also hyaloronate.
- 76. The composition of claim 68, comprising also an antibiotic.
 - 10.77, 10.5An aqueous composition; comprising the compositions; elevation 25

ionic constituents sodium, potassium, magnesium and bicarbonate, in water; - 0 f. - 0 f.i.ft

1600年(1818年) 11月1日 11月日 11月日

a buffer to maintain the pH of said composition within the human psychological range;
the carbohydrates pyruvate and lactate; and
glycine, proline, and glutamine:

5 78. The composition of claim 77, further comprising:

glutamine.

1 1. 18 (180) 180. A The composition of claim 77, further comprising taurine.

10 m 81.1 The composition of claim 77, comprising also hyaluronate.

82. The composition of claim 77, comprising also polyvinylpyrolidone.

The composition of claim 77, comprising also hyaluronidase.

The composition of claim 77, comprising also human serum albumin.

85. The composition of claim 77, wherein the form and concentration range, in

millimole per liter unless otherwise noted, of said components are as follows:

NaCi in the concentration range 75.0-105;

KCl in the concentration range 3.5-7.5;

to upolicity of the MgS04. A 150 in the concentration range 0.4-4.0;

NaHCO₃ in the concentration range 2.0-10;

20 the buffer MOPS in the concentration range 10-25.0;

while concentration range 0.5-2.0; The concentration range 0.5-2.0;

NaLactate (L-isomer) in the concentration range 5.0-2.0;

5.

15

NaPyruvate in the concentration range 0.1-1.0; minuted at virtual a Delta Sarpe A alanyl-glutamine in the concentration range 0.1-2.0; glycine in the concentration range 0.1-2.0: 19 metable of the order proline in the concentration range 0.05-2.0; The same and larger visa serine in the concentration range 0.05-2.0; and taurine in the concentration range 0.05-2.0.

- 86. The composition of claim, 85, comprising also hyaluronate in the concentration range 0.02-0.5 milligram/milliliter.
- The composition of claim 85, comprising also hyaluronidase in a concentration range of 0-80 IU/milliliter and human serum albumin in a concentration range 10 1.0-10 milligrams/milliliter.
 - 88. The composition of claim 77, comprising also at least one human reproductive cell selected from a group consisting of human gametes, human recommended by zygotes, and human embryos. A conficon more of
 - 89. An aqueous composition comprising the components: ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate, and KOLLE die Lercenbeilien ward 7 5-7.5: calcium, in water;
 - a buffer to maintain the pH of said composition within the human physiological 19 (20) maga, with arrange at at 20 Hall range;
- 13 the carbohydrates glucose, lactate, and pyruvate; which said 20 alanine, asparate, asparagine, glutamate, alanyl-glutamine, glycine, proline, serine cysteamine; The second of the

他看得她的主体,我们的人

arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, 5.6 - 165 threonine, tryptophan, tyrosine, and valine; and 3.3-5.6 the vitamins D-Ca Pantothenate, choline chloride, folic acid, i-Inositol, niacinamide, Mills (in pyridoxal HCl, riboflevin, and thiamine HCl. · 2003年 1895年 1896年 18 44.0 - 1.0 5 The composition of claim 89, comprising also human serum albumin (HSA) 90. :50-1.0 and hyaluronate. A see 146 P. Rear M. J. J. 12.05 - 30.24 Garage Leville The composition of claim 89, comprising also at least one component selected 91. 20-12 anten Tekel from the group consisting essentially of insulin-transferin selenium (ITS), insulin-like growth :1-1-10 - 12 May 1 22 factor (IGF), and epidermal growth factor (EGF). 1931 B = 113 B e am tett 92. The composition of claim 89, comprising also follicle stimulating hormone 10 R100.0 - 10.00 FR to 4 1 2 m (FSH). 0.03 - 0.02: 1 40 2-1 OH 0.0 HG 5 11.69 Pal The composition of claim 89, comprising also human chorionic 93. bits (Scittle 11 17 12) 3 5 872 1 gonadotrophin (hEC). The composition of claim 89, wherein the form and concentration range in 94. in legal and the state and the same the december of the of the state of the same in the same of the sa millimole per liter, unless otherwise noted, of the components are as follows: 15 80.0 - 100; bra A.M. - 3.5.27.5; © 325°0.05. - 1.5; NaH₂PO₄.2H₂O MgSO₄.7H₂O 0.2 - 4.0: 20 NaHCO, 15 - 30.0; m wyses sebastowone bas saCaSt.2H2Ohr \$2 tristale on it 0.842.8 it Glucose 0.5 - 5.5; NaLactates (Leisomer) of 2.0- 20.0; of common a NaPyruvate 0.01 - 1.0;25 Alanine 0.01 - 0.5: 6/15 Asparate 0.01 - 0.5; O Asparagine 0.01 - 0.5; Glutamate 0.01 - 0.5;

```
Alanyl Glutamine and Report 0.01102:0; Saluzgus
                                                                                                     Glycine
                                                                                                                                                                                                                0.01 - 0.5;
                                                                                                     Proline
                                                                                                                                            has write the saiston 1900 state and on the
                                                                                                     Serine
                                                                                                                                                                                                                0.01 - 0.5;
                                                                                                     Cysteamine de la ratio en (0.1 - 2.0 junto la 7 2.5
                                                                                                     L-Arginine-HCl
                                                                                                                                                                                                                  0.1 - 1.2;
                                                                                                     L-Cystine 2HCl [ part of the 0.05 at 0.25; differ for the
                                                                                                     L-Histidine-HCl-H2O
                                                                                                                                                                                                                  0.1 - 0.4;
                                                                        The Land of Land of the Land of the Co. 140.8; IT
         10
                                                                                                     L-Leucine
                                                                                                                                                                                                                  0.1 - 0.8;
                                                                                                     L-Lysine-HCl
                                                                                                                                                                                                                  0.1 - 0.8; เฮระแรงยโดยูอ์ โน ค
                                                                                                     L-Methionine
                                                                                                                                                                                                              0.05 - 0.25;
                                                                         L-Phenylalanine and applied 0.1 × 0.4;
                                                                                                     L-Threonine
                                                                                                                                                                                                                  0.1 - 0.8;
15 (15) Comment (18) I at L-Tryptophaneline The fluid reset of 19;00 query als moul
                                                                                                    L-Tyrosine 2Na
                                                                                                                                                                                                                  0.1 - 0.4;
                                                                                                     L-Valine (40%) and A favo 10.1 -0.8; but (25%) total
                                                                                                     D-Ca Pantothenate
                                                                                                                                                                                                          0.001 - 0.004;
Choline Chloride Chlo
                                                                                                     Folic Acid
                                                                                                                                                                                                        0.001 - 0.0045;
                                                                                                     i-Inositol
                                                                                                                                                                                                           0.005 - 0.02;
                                                                                                    Niacinamide
                                                                                                                                                                                                          0.004 - 0.016;
                                                                                                  Pyridoxal HCl
                                                                                                                                                                                            ____.0.002:≠0.<del>0</del>1‡
                                                                                                     Riboflavin
                                                                                                                                                                                                0.0001 - 0.0006; and
         25
                                                                                                                                                                                                          0.001 -.0.006) alford to analy
                                                                                                     Thiamine HCl
                                                                                    of the following of the Contraction of the Contract
                25. The composition of claim 94, wherein the form and concentration range, in
                                               through ger liter, anders conserve when is all the particular as all the conservers of the
                                milligrams per millimeter, of the components are as follows:
                                                                                             Human Serum Albumin
                                                                                                                                                                                                             1 - 10.9; and
                                                                                        . Hyaluronate
                                                                                                                                                                                  ⊙,⊱..... 0.05 ∔10.5.
                                                                                                                                                                                         必要少。。第25章
                                                                                                                                                                                                         Net City
                                                                                          15-30,3
         30
                                                          96.
                                                                                   The composition of claim 94, wherein the form and concentration range, in
                                                                                                                                                                                                           Figures:
                                nanograms per milliliter, of the components are as follows:
                                                                                                                                                                                                Statement State
                                                                                         ITS
                                                                                                                                                                                                                                                                                                                           -1 - 100;
                                                                                        GF-I
                                                                                                                                                                                                      10 - 1000; and
                                                                                        - FGF
                                                                                                                                                                                                ું <u>1000.</u>
                                                                                                                                                                                                   Shirt Bill
```

- 97. The composition of claim 94,7 comprising also the hormone follicle stimulating hormone in the concentration range 0.01-10 IU/milliliter.
- 98. The composition of claim 94, comprising also the hormone human chorinonic gonadotrophin in the concentration range 0.01-10 IU/milliliter.
 - 99. An aqueous composition, comprising the components:

ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate and calcium, in water:

a buffer to maintain the pH of said composition in the human physiological range;

carbohydrates glucose, lactate, and pyruvate;

alanine, asparate, asparagine, glutamate, alanyl-glutamine, glycine, proline, serine and

taurine;

5

hyaluronate; and

TOWN OF BUTCH WINDS AND PROPERTY.

angenia enniellen eligipaan vii 👢

human serum albumin (HSA). পুলি আনু ফৰ্মৰ কিন্তু চাইচেক্ট্ৰেলেলে লিক্ট্ৰেলিক কিন্তু কিন্তু কিন্তু কিন্তু কিন্তু

function (

100. The composition of claim 99, comprising also ethylenediaminetetraacetic acid

at the state of

THE PROPERTY OF MANY SECTION AND THE SECTION A

15 (EDTA).

101. The composition of claim 99, wherein the form and concentration range in millimole per liter, except as otherwise noted, of said components are as follows:

.शिव-दर्शकृतक्त्र नहीं इस की एक कर कि जिल्ला, स्टब्स कर के हैं है है है है।

ing beams, if years his last a	NaCl KClairlocation and	80.0 - 100;	
	NaH ₂ PO ₄ .2H ₂ O MgSO ₄ .7H ₂ O	0.05 - 1.5; ca. tadi 202 0.2 2.0; 4	
thus as ton privile and the		15.0 - 30;	•
	CaCl ₂ .2H ₂ O	0.8 - 2.8;	स्तारं द
100.6 8	Glucose	0.05 -5.0;	

18:00 % 7

3 : 1

3

٠.	West and S		NaLactate (Lisomer) NaPyruvate	U.1 - 1.U,	
		Plan Se	Note: Note: Albanine	apr est ar secusioni 0.01 - 0.5 ;	तुः, श्राणिकः । ते ।
i z e.'	Southfield Services	36 ⁴ 1	Asparate Asparagine		ĸ)
· 3			Glutamate Alanyl - Glutamine	90.01 +0.5;	हेतुकार केंग्रहण ह
			Glycine Charles and see Proline	0.1 - 1.0, 20.01 - 0.5; 0.01 - 0.5;	1 25
10	ps. 1 2 4 693	ering organization	Serine Taurine		Light is
				1 - 10.0; and	in the finding
1,775,7	10 M C 10 C	2.5	The second of th		•

102. The composition of claim 99, comprising also EDTA in the concentration range 0.005-0.20 millimole/liter.

on expensing that he command leaves leftly action brighted to the

- 103. The composition of claim 99, comprising also at least one human reproductive for a group consisting of human gametes, human zygotes and human embryos.
- 104. The composition of claim 99, wherein said composition is at least partially contained in a rigid housing.
- 20 105. An aqueous composition, comprising the components:

ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate, and the description of the descript

a buffer to maintain the pH of the composition in the human physiological range;

carbohydrates glucose, lactate and pyruvate; and

alanine, asparate, asparagine, glutamate, alanyl-glutamine, glycine, proline, and come.

106. The composition of claim 105, comprising also:

arginine, cystine? histidine, isoleucine? leucine, lysine, methionine, phenylalanine,

threonine, tryptophan, tyrosine, and valine.

12:19

107. The composition of claim 105, comprising also:

D-Ca Pantothenate, choline chloride, folic acid, i-Inositol, niacinamide, pyridoxal

5 HCl, riboflavin, and thiamine HCl.

108. The composition of claim 105, comprising also human serum albumin (HSA).

end version

109. The composition of claim 105, comprising also hyaluronate.

millimole per liter, except as otherwise noted, of said components are as follows:

10	:600 G NaCl	80.0 - 100;	
	; ₹0 0 ← ; KCl	30 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	
	등 ⁴ 실수 - NáH2PO4.2	2H2O 1005-15	
	₩## (MgSO4.7H)	20 6 302 40.	
	7610.6 (NaHCO3	^{1.369} √ √ 115 - 30 0·	
15	;:0 € - ⊆CaCl2.2H2 ()	
	bas (#600.0 Glucose	0.5 - 5.5	
	actate (1	L-isomer) 2.0 - 20.0;	
	NaPyruvate	0.01 - 1.0:	
	Alanine	0.01.0.0	
. 20 53/55555	what is AS is the Asparate 🗼	0.01 - 0.5;	<u> </u>
	Asparagine	0.01 - 0.5	
	Glutamate	79407 70.01 -0.5; 4914	
	Alanyl - Glu	itamine 0.01 2.0.	
(n in si bisi	nordayê bise di Giyeine sê'	out & a 120.0120.5	Ţ
25	Proline	0.01 - 0.5: and	
	Serine ###	် အားလုပ်ပြောက်ပါ -0.01/20.5 က ကသည	. "

nomental esta messi se cala generariano .PC: mileto la contra o rea e li

111. The composition of claim 105, wherein the forms and concentration ranges on a stronger to need the message married to grazel manager to a reasonable of the second manager.

in millimole per liter, except as otherwise noted, of said components are as follows:

		L-Arginine-HCl	- 一点語: 140.1.#1代2; 中華設定 0.05 - 0.25*
5- 4		L-Cystine 2HCl	0.05 - 0.25;
		L-Histidine-HCl _T H	2 0 – po <i>že</i> 0(1.±0:4; 4 %66% ೧೯೫೮
			0.1 - 0.8;
5	•	L-Leucine	vis mainig 0.1:=0.8; I = 501
		L-Lysine-HCl	0.1 - 0.8;
	्रीपञ्च । स्र	L-Methionine	∾
		L-Phenylalanine	0.1 - 0.4;
		L-Threonine	0.1 = 0.4;
10		L-Tryptophan	0.1 - 0.9;
	y gay a garage	L-Tyrosine 2Na	structure works 0.4; and still
		L-Valine	0.1 - 0.8.
	Street Ha	e cije gand i pecon jednice	and the section of the property of

112. The composition of claim 105, wherein the form and concentration range in millimole per liter, except as otherwise noted of said components are as follows:

D-Ca Pantothenate 0.001 - 0.004; 15 Choline Chloride 0.003 - 0.01; Folic Acid 77.1 (AC) T 0.001 - 0.0045; i-Inositol 0.005 - 0.02; Niacinamide 0.004 - 0.016; Pyridoxal HCl 20 5 FIRS, 0.002 - 0.01; 0.0001 - 0.0006; and Riboflavin Thiamine HCl ______ = = 0.001 - 0.006.

- 113. The composition of claim 105, wherein said HSA is in the concentration range of 1-10.0 milligrams/milliliter.
- 25 114. The composition of claim 105 wherein said hyaluronate is in the concentration range of 0.02-0.5 milligrams/milliliter.
 - 115. The composition of claim 105, comprising also at least one human embryos.

- contained in a rigid housing.
- TPA The composition of claim 105, wherein the concentration range of hyaluronate, in milligrams/milliliter, is 0.05-1.0.
 - 5 118 The composition of claim 105, wherein human serum albumin is omitted.
- 419. An aqueous composition for use in human in vitro fertilization, comprising the components:

ionic constituents sodium, potassium, magnesium, phosphate, bicarbonate, and calcium, in water,

- a buffer to maintain the pH of the composition in the human physiological range; and carbohydrates glucose, lactate and pyruvate.
 - 120. The composition of claim 119, comprising also human serum albumin.
- 121. The composition of claim 119, comprising also at least one additive selected from a group consisting of glycerol, ethylene glycol, dimethylsulfoxide, propanedial and surcrose.
- 122. The composition of claim 119, wherein the form and concentration range in millimoles perliter, unless otherwise noted, of said components, are as follows: NaCl in the range 75.0-105; KCl in the range 3.5-7.5; MgSO₄ 7H2O in the range 0.4-4; Na2PO4.2H2O in the range 0.1-1.5; NaHCO3 in the range 2.0-10; MOPS in the range 10.0-25; CaCl2.2H2O in the range 0.5-2.0;
- NaLactate (L-isomer) in the range 2-20; NaPyruvate in the range 0.1-1.0; and glucose in the range 0.5-5.5.

£.

	*	123.	The composition of claim 120, wherein the concentra	tion range of hur	man
	•		serum albumin is 1.0-10 milligrams per milliliter and the	1. 1 52-100, 1600	
	7.	124.	The composition of claim 121, wherein the concentration	on range of additi	ives
	-		glycerol, ethylene glycol, dimethylsulfoxide, and pror	ancdiol is 2 to 2	.0%,
5			and the concentration range for sucrose is 0.1 to 1 mol		Č
i . <u>t</u>		125.	A system for human in vitro fertilization, comprising	at least three cul	ture
	media,	includi	ing:	arcduror sup	
	op y'sel	(a)	a first culture medium, including:	\$127 0	
		ionic	constituents sodium, potassium, phosphate, magnesiu	m, bicarbonate,	and
10	calciu	n, in wa	ater; se pre ser e la conceu minare e la cinacial damies rib	$\mathbb{E}[H^{-1}\Omega]$	17.1
•			er to maintain the pH of said composition within the		gical
	range;	ji Ga (D	and the composite of the local and opposite of T	F 96	
State 1		the car	rbohydrates glucose, lactate and pyruvate; and	121	
-	the second	alanin	e, asparate, asparagine, glutamate, alanyl-glutamine, glyci	ne proline, serine	e and
15	taurine		•	े सम्बद्ध	d. E
3 J. 18	er in 1944	(b)	a second culture medium, including:	122	
f	 30.78 €	ionic	constituents sodium, potassium, phosphate, magnesi	um bicarbonate	and
	17261	m, in w	THE REPORT OF THE PROPERTY OF		
• 20 -	the R	a buff	er to maintain the composition at a pH in the human phy	ysiological range	
20	53.58.77.5	carbol	hydrates glucose, lactate, and pyruvate:		₹[i*]
	i Leta	alanin	e, asparate, asparagine, glutamate, alanyl-glutamine, glyci	ne, proline, serine	eand
	taurin		glacous in the coupe all 4.5.5		

human serum albumin (HSA); and

(c) rea third culture medium, including:

ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate, and

Real grade in San India in 1998.

5 calcium, in water;

carbohydrates glucose, lactate and pyruvate;

alanine, asparate, asparagine, glutamate, alanyl-glutamine, glycine, proline, and serine;

arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine;

D-Ca Pantothenate, choline chloride, folic acid, i-Inositol, niacinamide, pyridoxal HCl, riboflavin, and thiamine HCl;

human serum albumin; and

15 hyaluronate.

20

126. The system of claim 125, wherein said first culture medium includes:
ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate, and calcium, in water;

a buffer to maintain the pH of said composition in the human physiological range; the carbohydrates glucose, lactate, and pyruvate; and alanine, asparate, asparagine, glutamate, glycine, proline, serine, and taurine.

127. The system of claim 125, wherein said first culture medium includes:

THOSE OF STREET &

.

ionic constituents sodium, potassium, magnesium, phosphate, bicarbonate, and calcium, in water;

a buffer to maintain the pH of the composition in the human physiological range;

the carbohydrates glucose, lactate, and pyruvate;

5 human serum albumin; and

at least one additive selected from a group consisting essentially of glyccrol, ethylene

glycol, dimethylsulfoxide, propanediol and sucrose the desired missions.

AND THE WAS LIGHT OF STANDING OF STANDING THE PROPERTY OF STANDINGS OF

and the state of t

HCL abothwa sed him was UC

and worked research

abrion bigi

See The system of chains 125, when it wishes no here to the reduction of the

and the control of the control of the control of the property and the control of the control of

groups in accounting

(1)

the control and the first care of the planch being and first to Thy oil, named an of sullarly a

व्यक्तिकार या सम्बन्धित विकास का तम्म हार स्थान है। इस्तारिक प्रतास कर जिल्ला का कार्य है।

normal to the an are the affine prime of a thinker or an area of the

WO 00/32140(442) (2.27) (3.87) (3.87) (3.87) (3.87) (3.87)

of the control of the early and the control of the

PCT/US99/28408

STATEMENT UNDER ARTICLE 19(1)

An amendment to PCT Application PCT/US99/28408 has been submitted simultaneously with this Statement. The amendment replaces claims 1, 36 and 40 with new claims that seek to respond to the observations made in the International Search Report. Claim 1 has been amended to make clear that the embodiment referenced in Claim 1 relates to human in vitro fertilization, as opposed to animal in vitro fertilization, as in Abeydeera, et al., and to indicate that the core group of salts comprises at least two different salts. Additionally, claim 40 has been amended to state that the first support medium comprises three components, glucose, lactate and pyruvate, instead of the two components HSA and hyaluronate.

international description of the second seco

Similar Mar English of the English of the

HILD GLAD TO BE STONE OF THE BUILD

 $\{A_i: A_i \to A_i\}$

1 有 34. 3**3**M 1 14.

T. W. S. S. FELL A

THE SHIP SHE

C 45 (63)

. The expression constraints with the last policies of 0.09 . With a ratio 20.7%

16 Sec. - 17 1

the interest transmit of many of the state of the

jaformallokobant ilminetlinuk 130 PC (Getting Folker)2000 (17 Tolkette ilikeliti) uli a

For exceeding which and other differential messes, it is sea the feet in the second of the second of

MANUSTREET, FOR CONTROL MORE COME. BROWN AND LONGOR FOR THE MODELL TO

The wide wishes the set of contents of the set of the s

CORRECTED VERSION

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date 8 June 2000 (08.06.2000)

(10) International Publication Number WO 00/32140 A1

(51) International Patent Classification7: A01N 1/02

A61F 5/58,

(21) International Application Number: PCT/US99/28408 ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW.

(22) International Filing Date:

(25) Filing Language:

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent English (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, TE, IT, LU, A MARIE TO A DESCRIPTION MC, NL. PE. SE), OAPI patent (BF, BJ, CF, GG, CI, CM, English GA, GN, GW, ML, MR, NE, SN, ITD, TG)

(26) Publication Language: 群选位 的复数形

with the balas transfer of the enquiror the lies (30) Priority Data:

109/201,594 30 November 1998 (30.11.1998) US With international search report. With amended claims and statement.

(71) Applicant: IVF SCIENCES COLORADO, INC. [US/US]; Suite 300, 799 E. Hampden Avenue, Englewood, CO 80110 (US).

Date of publication of the amended claims and statement: 14 September 2000

(72) Inventors: GARDNER, David, K.; 9927 Clyde Circle, Highlands Ranch, CO 80126 (US). LANE, Michelle; 1661 West Canal Circle, #324, Littleton, CO 80120 (US).

(48) Date of publication of this corrected version: 7 December 2000

(74) Agents: FISCHMANN, Kent, A. et al.; Holme Roberts & Owen LLP, Suite 4100, 1700 Lincoln Street, Denver, CO 80203 (US).

(15) Information about Correction: see PCT Gazette No. 49/2000 of 7 December 2000, Section

(81) Designated States (national): AE, AL, AM, AT, AU, AZ,

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE,

(54) Title: SYSTEM AND SEQUENTIAL CULTURE MEDIA FOR IN VITRO FERTILIZATION

(57) Abstract: Instead of immersing human reproductive cells in a single culture medium throughout the various procedures used in IVF, a process is provided by which the reproductive cells may be moved through a sequence of distinct culture media as the various IVF procedures are carried out. In one implementation, the culture media specifically formulated to provide a physical environment similar to that found within the female reproductive tract and conducive to growth and development of human reproductive cells during the various stages of the IVF process. In this regard, specifically formulated culture media can be applied to support the reproductive cells in one or more of the following procedures: oocyte retrieval and handling; oocyte maturation; ordinary fertilization; oocyte, zygote and embryo examination and biopsy; embryonic development to the eight-cell stage; embryonic development to the blastocyst stage; embryo transfer; and cryopreservation.

: ' WO 00/32140

5

10

15

20

25

30

SYSTEM AND SEQUENTIAL CULTURE MEDIA FOR IN VITRO FERTILIZATION

FIELD OF THE INVENTION

and, in particular, to a sequential culture media system and process to be used in oocyte retrieval, handling and maturation, sperm preparation, fertilization, embryo development and transfer, and cryopreservation. The invention provides the gametes, zygote and developing embryo with a physical environment adapted to their physiological needs, so supporting their normal growth and development in vitro and increasing the likelihood of successful pregnancy.

The allieur states again BACKGROUND OF THE INVENTION With a great and the

In vitro fertilization seeks to duplicate, to a large extent, the conditions and processes normally occurring within the female reproductive system that are necessary to pocyte development, fertilization and early embryonic development. In the clinic and laboratory, IVF involves several discrete procedures, such as collection of the oocytes from the ovary of the mother, preparation of the sperm, fertilization, and, once fertilized eggs are identified, a period of early embryonic development, and then transfer of the embryo to the mother's uterus. Each of these steps can take place over extended periods of time; during which the individual cells involved have a continuing need for nutrients, and are subjected to significant stress as a result of clinical manipulation and changed environmental conditions.

During IVF, a culture medium is ordinarily used as a substitute for the fluid secreted by the female reproductive tract that would ordinarily surround the gametes, zygote, and developing emerge. Most laboratories carrying out IVF use a single culture medium throughout the various procedures involved. In a number of laboratories, there has been a tendency to use tissue culture media for IVF procedures, such as Ham's F-10, which is formulated to support somatic cell growth, not gamete or embryonic cell growth. Tissue culture media generally are complicated systems, containing an array of amino acids, vitamins and other constituents. They can contain components that significantly impair embryonic development and viability after transfer. Further, to the extent tissue culture media contain components that are

WO 00/32140 CT/US99/28408

generally needed by the gametes and the embryo, the media are not formulated to provide the components at levels appropriate to support healthy gamete and embryonic development.

5

10

15

20

25

30

Other laboratories have used simple culture media, consisting of balanced salt solutions supplemented with carbohydrate energy sources such as glucose, pyruvate and lactate. Examples include Earle's, T-6, and human tubal fluid (HTF). These media generally lack certain key components found in the female reproductive tract, such as non-essential amino acids, and their constituents are not formulated in concentrations that meet the specific changing needs of the gametes and developing the embryo at various stages of their development.

 $\{ \cdot \}$

1

Č.

The two types of culture media commonly used for IVF generally are only capable of supporting embryonic development to the eight-cell stage. Such media are ordinarily not capable of supporting and producing a viable blastocyst stage embryo, complete with an epithelium and competent inner cell mass. Accordingly, embryo transfer, the return of the fertilized oocyte to the uterus of the mother, usually occurs at around the four-cell stage (day two) or around the eight-cell stage (day three). This is a time when the four- or eight-cell embryo would not typically have arrived in the uterus of the mother, if fertilization had occurred in vivos. Embryo transfer at this time involves placing the cleavage stage embryo in an environment oriented to a blastocyst stage embryo. The cleavage stage embryo must then undergo further development in a non-homologous environment to reach the blastocyst stage, in which the embryo has trophectoderm cells capable of implanting in the uterine lining

Recent research and human trials have led to the development of two new culture media, G1 and G2, which represent significant advancements in adaptation of culture media to the physiological needs of the cleavage stage embryo and the embryo in the eight-cell through blastocyst stage of development. These media are described in the following publications: Barnes, Crombie, Gardner, et al. Blastocyst

Development and Birth After In-vitro Maturation of Human Primary Oogytes, to the control of the control of the cleavage stage embryo and the embryo in the following publications: Barnes, Crombie, Gardner, et al. Blastocyst

Development and Birth After In-vitro Maturation of Human Primary Oogytes, to the control of the control of Human Reproduction, volume 10, no. 12, pp. 3243-47 (December, 1995); Gardner and Lane, Culture and Selection of Viable Blastocysts: A Feasible Proposition for Human IVF?, Human Reproduction

Update, Vol. 3, No. 4, pp. 367-82 (1997); Gardner, Vella, Lane, et al. Culture and

²⁶ WO 00/32140 PCT/US99/28408

Transfer of Human Blastocysts Increases Implantation Rates and Reduces the Need for Multiple Embryo Transfers, Fertility and Sterility, Vol. 69, No. 1, pp. 84-88 (January 1998). Use of these media, and particularly the G2 medium, supports the growth and development of viable blastocyst stage embryos in vitro. Accordingly, the development of these media paves the way for new approaches to embryo transfer to the uterus at the blastocyst stage, when the embryo is most adapted to surviving in the uterine environment and has developed structures and capabilities required for implantation to take place. Research utilizing the G1 and G2 media, and embryo transfer at the blastocyst stage, suggests that these media contribute to higher pregnancy rates, and reduces the need for transfer of multiple embryos and the risk of multiple births. Neither of these media, however, is optimized for supporting the gametes, oocyte maturation, of fertilization.

5

10

15

20

25

30

THE PROPERTY OF THE INVENTION OF THE PLANT OF THE PROPERTY OF THE INVENTION OF THE PROPERTY OF

ran stationala i agranista 🎎 🙉 gradisia a 🗴 🔾 🔾 🖂 🖂 🖂

It has been recognized that IVF processes may be improved by providing specific media and media sequences for supporting gametes, zygotes and developing embryos relative to various phases of the IVF process. In certain respects, such media and sequences better reflect in vivo development. Within the female reproductive system, the oocyte is developed within and released from the ovary during ovulation, and proceeds through the oviduct towards the uterus. During this journey, it experiences a dynamic physical environment. The fluid of the oviduct contains a number of components that provide nourishment to the oocyte and its surrounding cumulus cells, and that also appear to interact with the oocyte and its cumulus cells, so stimulating development. Similarly, the fluid of the female reproductive tract provides nourishment to sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct and enters the uterus approximately three days later, undergoing internal mansformation and experiencing a changing environment.

As the zygote travels, cell division, or cleavage, occurs as well as significant developmental changes. The cells of early embryonic development have different capabilities and nutritional needs from those of later embryonic development prior to

implantation. The zygote and cleavage stage embryo (up to the eight-cell stage) are characterized by low levels of bigsynthesis, low respiratory rates, only limited ability to metabolize glucose, and a capacity to utilize pyruvate. As the embryo develops. and genome activation occurs, the embryo gains an increased capacity to utilize glucose. At the blastocyst stage of development, when the embryo is entering and a within the uterus, the embryo's metabolic system has developed and the embryo has a substantially greater capacity to use and need for glucose, and less need for pyruvate. The makeup of the fluid surrounding the developing embryo in theifemale in the property reproductive tract seems to be tailored to these changing needs; in the oviduct at the time when the oocyte and developing embryo are present, relatively low levels of glucose are found, while pyruvate concentrations are high; at the time the embryo enters the uterus, glucose reaches its highest level and the pyruvate concentration is comparatively low. Cleavage stage embryos, like the oocyte, are susceptible to loss of amino acids through their cell membranes when surrounded by an environment having a low concentration of such factors. Such loss of internal amino acids can have devastating effects. Again, as if in response to these needs of the osmolyte had the sensitive oocyte and cleavage stage embryo, the female reproductive tract typically has high levels of specific amino acids that are very similar to those found in the a base oocyte and cleavage stage embryo. Wat his a new his government a cook with the work

20

15

5

10

In view of the foregoing, an important object of the present invention is to the further improve and enhance the culture of human reproductive cells in vitro. The invention is intended to promote the health and viability of the garnetes, expote and embryo at various stages of the IVF process, thereby improving the dverall efficiency: of the IVF process and increasing pregnancy rates.

1)5

11

€ :

25

30

In general, the present invention involves the application of separate media or a specifically formulated to meet the physiological needs of the gametes, zygote and/or a developing embryo in various stages of their development, and to support the application processes necessary to accomplish fertilization and embryonic development invitto. The present invention also generally contemplates a sequential culture media system in which the separate media utilized have integrated formulations, intended to an another during the IVF process.

% WO 00/32140 PCT/US99/28408

In one aspect of the present invention, an oocyte retrieval and handling medium is provided for use in the clinical procedure of retrieving the oocyte from the mother. The medium may be used for flushing, washing and holding the oocyte during the process of removing the oocyte from the mother's ovary, and for storing the oocyte for a period prior to fertilization. An optional use of the medium envisioned by the invention is with procedures where handling or manipulating the oocyte, zygote, or embryo is necessary, such as examination of the oocyte to determine whether fertilization has occurred, or examining the embryo to determine the progression of its development. The present invention includes use of an oocyte retrieval and handling medium comprised of water, ionic constituents, and a buffer. Preferably the buffer used in the medium is 4-Morpholinepropanesulfonic acid (MOPS) or N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES). In addition, the medium may be supplemented with the carbohydrates glucose, lactate and pyruvate. The medium may be supplemented with non-essential amino acids. An optional formulation of the medium, lacking calcium and magnesium, may be used in biospsy procedures. Another optional formulation of the medium includes antibiotics, such as penicillin and/or streptomycin, to destroy bacteria that might be introduced into the medium during the process of oocyte collection.

5

10

15

20

25

30

Another aspect of the present invention involves the provision and use of an oocyte maturation medium, for example, in circumstances where the oocyte is removed from the mother before it is mature. An example of a situation where application of this medium may be desired arises when it is necessary to treat the oocytes collected from the mother with hormones in vitro due to the mother's intolerance of such normones. The invention contemplates holding the oocytes in the maturation medium for a period following collection of the oocytes, to promote development prior to fertilization. An optional use of the maturation medium in accordance with the invention is for collection, although the most cost effective approach will normally involve use of the retrieval and handling medium for initial flushing, washing, collection and storage, and then transfer to the maturation medium for an extended period prior to fertilization. The present invention contemplates use of a maturation medium comprised of water, ionic constituents, and a buffer. Preferably, the maturation medium is supplemented with the carbohydrates glucose,

13.1

5

10

15

20

25

30

lactate and pyruvate. Specific formulations in accordance with the present invention may involve successive supplementation of the medium with one or more of the following: non-essential amino acids; essential amino acids; cysteamine; human serum albumin (HSA) and hyaluronate; one or more growth factors such as insulin transferin selenium (ITS), insulin-like growth factor (IGF), and epidermal growth factor (EGF); and hormones follicule stimulating hormone (FSH) and human chorionic gonadotrophin (hCG).

Another aspect of the invention involves the provision and use of a sperm preparation and fertilization medium. This medium may be applied to wash, prepare, and store sperm, to store the oocyte in the period prior to fertilization, and to serve as the medium in which the sperm and oocyte are placed together and fertilization occurs. The present invention contemplates use of a sperm preparation and (2000) fertilization medium that includes water, ionic constituents, and a buffer. Preferably, the medium contains an elevated concentration of sodium, as compared to the oocyte retrieval and handling medium, to promote sperm function and fertilization. In addition, the medium may be supplemented with an elevated phosphate concentration, as compared to the oocyte retrieval and handling medium. Even more preferably the medium is supplemented with the carbohydrates glucose, lactate and pyruvate Specific formulations may involve supplementation of the medium with one or more of: bicarbonate; glutathione to promote sperm head decondensation; non-essential amino acids; HSA and hyaluronate; and antibiotics such as penicillin and application was myem. 20. 2003 - D y never al 81 D. d. d. 2004. Universide of American entitlem establish no invitigat streptomycin.

A further aspect of the invention relates to a medium for intracytoplasmic sperm injection (ICSI) and related methodology. The ICSI procedure may be necessary where there are obstacles to normal fertilization, such as a thickened zona pellucida on the oocyte hindering sperm head penetration. ICSI involves removal of the cumulus cells and injection of the sperm into the oocyte, ordinarily through a glass pipette. The invention contemplates placing sperm in the ICSI medium, capturing the sperm by drawing the medium containing sperm into the pipette, inserting the pipette containing medium and sperm into the oocyte, and, following insertion into the oocyte, transferring the medium containing sperm from the pipette into the oocyte. The ICSI medium used in the present invention includes the constituents water, ionic

WO 00/32140 PCT/US99/28408

constituents and a buffer. Preferably, in the present invention the medium lacks phosphate. More preferably, the buffer used in the medium is MOPS or HEPES. Additionally, the medium may be supplemented with the carbohydrates lactate and pyruvate and the medium may be further supplemented with one or more of the non-essential acids most abundant in the oocyte: glutamine, glycine, proline, serine, and taurine. In one formulation, the ICSI medium used is supplemented with hyaluronate or polyvinyipyrolidone (PVP) to slow or immobilize the sperm so that they may be captured by pipette for the ICSI process. Further, an alternative formulation of the ICSI medium referred to as denuding medium used in the invention includes hyaluronidase, which is included in the portion of the medium used to denude the oocyte prior to the ICSI process.

5

10

15

20

25

30

Another aspect of the present invention involves the provision and use of a medium for supporting initial cell cleavage and embryonic development following fertilization, the medium herein referred to as G1.2. The invention contemplates washing the inseminated oocyte and zygote in the medium and placing the zygote in the medium for a period of about 48 hours to support cell cleavage and development through about the eight-cell stage. The present invention involves use of a medium that includes the constituents water, ionic constituents, and a buffer. Preferably, the medium is supplemented with the carbohydrates glucose, lactate, and pyruvate. The medium may also be supplemented with non-essential acids. Specific formulations in accordance with the invention may include one or more of the following supplements: EDTA, HSA, and hyaluronate. The form of glutamine used in the medium is preferably alanyl-glutamine, which is particularly stable and less prone to breakdown to the waste product ammonium, which is toxic to the developing embryo.

A further aspect of the invention involves the provision and use of a second medium for embryo development, herein referred to as G2.2. The invention contemplates placing the embryo in the G2.2 medium for a period of about 48 hours, preferably at or after the eight-cell stage, and continuing through the blastocyst stage of development and up to the point of embryo transfer. This medium is specifically adapted for and has as its preferred use support of the embryo from the eight-cell stage through the time at which implantation occurs, in tandem with the use of G1.2 for initial embryonic development. The invention involves a G2.2 medium that

);

5

10

15

20

25

30

supplemented with the carbohydrates glucose, lactate and pyruvate. More preferably, as compared to medium G1.2, medium G2.2 is supplemented with depressed levels of lactate and pyruvate, and elevated levels of glucose. Additionally, the medium may be supplemented with the non-essential amino acids, except taurine. Specific formulations in accordance with the present invention involve supplementing the medium with one or more of: essential amino acids, which stimulate development of the inner cell mass of the blastocyst; vitamins, which further facilitate the function of the blastocyst; HSA; and hyaluronate. An important aspect of the G2.2 medium, in all formulations, is the absence of EDTA.

Another aspect of the invention is the provision and use of an embryo transfer medium. The invention contemplates that this medium will be used as a carrier for the embryo when it is transferred back into the mother. The invention may involve the same formulations of the medium for embryo transfer as are used with medium.

G2.2. More preferably for embryo transfer, however, the formulation of G2.2 is supplemented with a higher concentration of hyaluronate, which supports implantation of the embryo in the mother's uterus.

A further aspect of the invention is the provision and use of a medium for cryopreservation of the embryo and/or oocyte. The invention contemplates that the embryo may be placed in the medium at either the one- to eight-cell stage or eight-cell to blastocyst stage, and then frozen and stored in the medium. The invention also contemplates that the medium may be used for cryopreservation of the oocyte. The cryopreservation medium contains ionic constituents, and a buffer. Preferably, it contains the MOPS or HEPES buffer. More preferably, it contains the carbohydrates lactate, pyruvate and glucose. Even more preferably, it contains HSA Most preferably, the medium contains certain additives such as glycerol, ethylene glygol, DMSO, and/or sucrose.

According to a further aspect of the invention, different media are used for two different phases of the IVF process, such as oocyte collection and maturation, sperm preparation, fertilization, embryo development and/or embryo transfer. One associated process involves obtaining a gamete from a first medium and introducing the gamete into a second medium different from the first medium, wherein

PCT/US99/28408

fertilization occurs in the second medium. The step of obtaining a gamete from a first medium may include extracting an oocyte from an oocyte collection medium or oocyte maturation medium as described above. Additionally or alternatively, the step of obtaining may involve extracting sperm from a sperm preparation and fertilization medium as described above which, in turn, may be different from the oocyte medium. The step of introducing the gamete into the second medium may involve introducing the sperm and/or oocyte into a fertilization medium, or injecting sperm into an oocyte contained in the second medium. The various media may have integrated formulations for minimizing trauma to the reproductive cells.

10

15

20

5

Another associated process in accordance with the present invention involves obtaining a zygote or embryo from a first medium wherein fertilization has occurred and introducing it into a second medium different from the first medium for a first growth phase. The first medium may be a fertilization medium as described above and the second medium may be the G1.2 medium as described above. The second medium may be used for supporting initial cell cleavage and embryonic development. The method may further involve transferring the resulting embryo from the second medium to a third medium for a second growth phase. The third medium may be a G2.2 medium as described above.

A further associated process in accordance with the present invention involves obtaining an embryo from a first medium and introducing the embryo into a second medium different from the first medium for transfer of the embryo into the mother for implantation. The first medium may be a G2.2 medium as described above and the second medium may be an embryo transfer medium as described above.

BRIEF DESCRIPTION OF THE DRAWING

25

For a more complete understanding of the present invention and further advantages thereof, reference is now made to the following detailed description taken in conjunction with the drawings, in which:

Figure 1 is a flowchart illustrating an IVF process in accordance with the present invention.

30

The following description discloses the composition of various culture media? in accordance with the present invention that are particularly adapted for use with con-IVF. Each of these media is specifically formulated to meet the physiological needs. of the gametes, zygote and developing embryo at key points in the reproductive addition process. Also disclosed is a sequential culture media system. While each of the short separate media could be used independently, the media also may be formulated. together as a system, sharing a core group of ionic and non-essential amino acid constituents, with the objective of minimizing trauma to the oocyte, and the resulting zygote and embryo, as they are moved from one medium to another. The following to description also discloses methods of using the media and the sequential culture media system in various clinical and laboratory procedures by which IVF is carried also out, as well as methods of making the culture media on broader and it perfects out has greet that see. The first analysis may be a fe thireties and a greet part had A. The Composition of the Sequential Culture Media, the artificial transfer in become many for Description Occyte Retrieval and Handling Medium of Squar with the role of the last of A preferred oocyte retrieval and handling medium is an aqueous solution and the second solution and th comprised of the ionic components sodium, potassium, phosphate, magnesium, and alege

61

21

20

20

5

10

15

comprised of the ionic components sodium, potassium, phosphate, magnesium, and business bicarbonate, and calcium, to maintain an osmotic environment that does not stress the coocyte, and a buffering system, preferably MOPS or HEPES, to maintain the pH of the medium within the physiological range of 7.3 to 7.4. The ionic components are not supported as a configuration of the medium within the physiological range of 7.3 to 7.4. The ionic components are not supported as a configuration of the medium within the physiological range of 7.3 to 7.4. The ionic components are not supported as a configuration of the physiological range of 7.3 to 7.4. The ionic components are not supported as a configuration of the physiological range of 7.3 to 7.4. The ionic components are not supported as a configuration of the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological range of 7.3 to 7.4. The ionic components are not supported by the physiological rang

SMET DESCENDING PORTED RATE DRAFF DAY

For choice complete the eighth ling of a present recention and the first advantion and the first advantages thereof. After deep in one in the following families dissocitation of the instance of the computation of the instances, in which is

e finally on the course of the property of the

DEWLERVE SEE DING THE OVERLY OF

included in the preferred amounts depicted in column A of Table 1, and may be included in amounts described in the ranges depicted in column B of Table 1.

S TO SEE IN CONTROL OF THE PARTY OF THE PART Composition of Oocyte Retrieval and Handling Medium*

5	Component A Component	В
	Component A respector conditions of American Light Most Preferred	Preferred
	Concentration	Range
	NaCl Office the content of the solution of the	
	KCl. whole specified distanced year vigure of the 5.5 expression of the	3.5 -7.5
	NaH ₂ PO ₄ .2H ₂ O 0.25 MgSO ₄ .7H ₂ O ²¹ (1.82.020) 10 (1.22.22)	0.05 - 1.5
10		
10	Na共COing and how as and parenting on the state of the first and being a record of the MOPS / HEPES 20	
	ofference that the second of t	10.0 - 25.0 0.8 - 2.8
	setudo placidos edicam ou il centro en internacional particolor de la color de la color de la color de la color	
	Glucose NaLactate O.5 NaLactate O.5 (L-isomer) O.5 O.5 O.5 O.5	0.05 - 5.0
15		
1-5	NaPyruvate, Immedicional in Informity his page 0.32 per in the many in the	0.1 - 1.0
		laws. :
	Alanine (con azoria , sai) (ala), onlo i ser a la coma 10.1 (said do azida said said said	0.01 - 0.5
	Asparate (asp) 0.1 Asparagine (ash)	0.01 - 0.5
	Glutamated and support (glu), Leading the second of the grant of the problem of the	0.01 - 0.5
20	Alanyl - Glutamine (ala - gln) 0.5	0.01 - 0.3
	Alanyl - Glutamine (ala - gln) 0.5 Glycine (gly) 0.1	0.01 - 0.5
	Proling To total of i(pro) and execution for the O:1 con fing the many of the property	0.01 - 0.5
	Serine Taurine (ser) (tau) to interest along the label of the series of	0.01 - 0.5
	Your strangenesses Lighter within the Lock of and the land of the	
25	* Concentrations are in millimoles unless otherwise indicated; the medium is	· (1)
	uquoous.	
	re in a od yere delika to Dean ea for neder on it envitages at each one by	1.7.7.
	It should be noted that Table 1 and the other tables presented in this section also	y d
	describe the preferred form of the components used to make the respective culture and among the first and survey of the components used to make the respective culture and a first and a survey of the components are to the components of the respective culture.	;
	media in practice. The MOPS buffer has not been used before in IVF procedures,	and
30	is preferred because it is not known to exhibit any toxic effects to reproductive cel	
•	and does not require maintenance of a CO ₂ atmosphere above the medium. HEPE	S
	may also be utilized, although some research indicates a possible toxicity to	
	reproductive cells. Table 1 depicts the preferred amount and ranges for the MOPS	or _n
	HEPES buffer, although other buffering systems might be used. For example, a	
35	bicarbonate buffering system may be used because it is compatible with human	<i>(2.1</i>)

reproductive cells. Such a system would not ordinarily be practical with oocyte collection, because it requires the maintenance of elevated levels of CO₂ in the atmosphere surrounding the medium, which is ordinarily accomplished by use of a gassing incubator system that maintains a 3%-10% CO₂ atmosphere. Oocyte collection is a clinical procedure, in which it is typically not possible to maintain an elevated CO₂ atmosphere. In some clinical environments, such as where a humidicrib is available, it may be possible to perform oocyte collection in an elevated CO₂ atmosphere, and a bicarbonate buffer accordingly may be used. In accordance with the present invention, any buffering system used preferably maintains its buffering qualities during exposure of the medium to the atmosphere, and as well is preferably compatible with and not toxic to human reproductive cells.

5

10

15

20 -

25

30

The oocyte retrieval and handling medium also includes the carbohydrates glucose, lactate, and pyruvate, at levels similar to those found in the female reproductive tract at the corresponding point of ovulation. The preferred amounts and ranges in which these are found in the medium are depicted in Table 1. In addition, the preferred medium contains Eagle's non-essential amino acids (i.e., those not state) required for the development of somatic cells in culture) alanine, aspartate, asparagine, glutamate, glycine, proline, serine, and taurine, plus glutamine in the form of alanyl-glutamine, at levels similar to those found in the female reproductive system and in the oocyte. The preferred amounts and ranges are depicted in Table 1. The field wine? inclusion of non-essential amino acids and alanyl-glutamine in the medium is SHIP THE !! important to preventing osmotic shock; a medium lacking these components may drain the oocyte of its internal pool of amino acids, resulting in considerable intracellular trauma. An optional formulation of the medium which may be used in biopsy procedures, omits calcium and magnesium. Another optional formulation of the medium may include one or more antibiotics, such as penicillin and streptomycin, to destroy any bacteria that might be present around the oocyte or that might be introduced through the clinical procedure of oocyte removal. widthauf in multiger, and also in create of a lower at the arresponding an apparation about the

2. Occyte Maturation Medium

The oocyte maturation medium is adapted for use with immature oocytes.

Oocyte maturation is typically used with mothers who are unable to withstand the

Title of the manufacture as the contract of the contract of the manufacture of the contract of

33

hormonal treatment ordinarily employed in IVF. Oocyte maturation generally involves treating the immature oocytes in vitro with the hormones follicle stimulating hormone (FSH) and human chorionic gonadotrophin (hCG) rather than injecting these hormones into the mother. The preferred medium is an aqueous solution that contains ionic constituents similar to those used in the oocyte retrieval and handling medium, at similar concentrations, although the magnesium level is increased and the calcium level decreased to maintain a 2:1 magnesium to calcium concentration. A buffer is included in the preferred medium to maintain a physiological pH. Because oocyte maturation ordinarily occurs in an incubator or isolette in which an elevated CO₂ atmosphere can be maintained, a bicarbonate buffering system is preferred. Other buffers may be used, provided they are compatible with the oocyte and other components of the medium. Table 2 provides the most preferred amounts of each of these components, as well as the preferred ranges of these components.

25

. 1

: 1.

.0

 \mathcal{L}°

 $i \in \mathbb{C}$

5950

PD:

73.00

11 (11)

 $Q_{i} \mapsto \{i\}^{2} Q_{i}$

Exclusion

6,00,3

100

1969. 11

A-1,2002 (.2)

hard work .

Long: All

 $\mathbb{L}^{(p_1, \frac{1}{p_2}, \frac{1}{p_2}, \frac{1}{p_2})}$

12 27 ()

W. Natio

3-1.00 mg 3.0

1.22

ji ku i negi ka Ligara Ali

ಪರ್ನೆಯಾಗು

7 Sola in Trut

元 410 日 十

1.1

, however,

第十年。1847年第

1 Hames - .

"THE WALL OF

Mr. Lat gal

370

€ -

60-10

0.05 - 6.2

8.3 - 1.0

8.6 - 1.3 8.6 - 1.1

2.0 m 39

0.1 - 0 2

5000 - 1000

3.503 + 0.61

9-401 - 101.0

5.605 - 0.02

616 N - 408 B

100分。10年代

5001.5 - 1330.6

30 C = 300.0

 $\mathfrak{t}^{1}(f,\xi)=\frac{1}{2}$

200-200

フルチュリ 10-10-10-01

633 C.

18 - 10/3

ta lu repares mario tra principion pull di recomo fin espire je fit assi espit espi

Table :

	Composition of Oocyte Maturation Medium*	
	Comp nent	
	Most Professed	Pr ferred
	C ncentrati n	Range
	NaCle 1900 And the second seco	तर्ग केल्प नार्गाम हत्ते । 100
5		
3	KCl NaH ₂ PO ₄ .2H ₂ O 0.25	0.05 - 1.5
	MgSO ₄ .7H ₂ O managed and the state of the	
	NaHCO 25	15 - 30.0
	CaCl_2H_O. A Third to be subjected to the subject of the subject o	21 NATIONAL NO.85-2.8
10		
	NaLactate (L-isomer)	2.0 - 20.0
•	NaPyruvate 0.1	0.01 - 1.0
	Alemine 2009 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	0.01 - 0.5
	Asparate Asparagine Asparagine O.1 O.1 O.1	not arm, everal-pa 0.01, - 0.5 [F]
15	Asparagine 0.1	0.01 - 0.5
	Glutamate Quality of the state of the safety of the best word for	.න යේ දුණය ස ෑ0.01 ේ 0.5
	Asparagine Glutamate Alanyl - Glutamine 0.1 1	0.01 - 2.0
	Glycine 13 grant an harring quality of the 12 SO.1 with the same and	11.3 RV 43443 RV 0.04 - 0.5
	Proline Serine O.1 Serine O.1 Serine	
20	 	
	Cysteamine 0.5	0.1 - 2.0
	L-Arginine-HCl 0.6	0.1 - 1.2
	L-Cystine 2HCl 0.1	0.05 - 0.25 0.1 - 0.4
0.5	L-Histidine-HCl-H2O 0.2 L-Isoleucine 0.4	0.1 - 0.4
25	1-150/cuonio	0.1 - 0.8
	E-Eddine	0.1 - 0.8
	E Djoine 1101	0.05 - 0.25
	D Modification	0.1 - 0.4
30	L-Phenylalanine 0.2 L-Threonine 0.4	0.1 - 0.8
30	L-Triptophan 0.5	0.1 - 0.9
	L-Typtophan L-Typtosine 2Na 0.2	0.1 - 0.4
	L-Valine 0.4	0.1 - 0.8
	D-Ca Pantothenate 0.002	0.001 - 0.004
35	Choline Chloride 0.007	0.003 - 0.01
<i></i>	Folic Acid 0.0023	0.001 - 0.0045
	i-Inositol 0.0111	0.005 - 0.02
	Niacinamide 0.0082	0.004 - 0.016
	Pyridoxal HCl 0.0049	0.002 - 0.01
40	Riboflavin 0.0003	0.0001 - 0.0006
	Thiamine HCl 0.003	0.001 - 0.006
		. 100
	HSA 5mg/ml	i - 10.0 0.05 - 0.5
	Hyaluronate 0.25mg/ml	0.03 - 0.3
	ITS 10ng/ml	1 - 100
45	ITS 10ng/ml IGF-I 100ng/ml	10 - 1000
73	EGF 100ng/ml	10 - 1000
	FSII 0.1U/ml	0.01 - 10
	hCG C.1U/ml	0.01 - 10
	* Concentrations are in millimoles, unless otherwise indicated; the medium is aque	eous.

The carbohydrates glucose, lactate and pyruvate are also included in the preferred maturation medium. Because of the presence and importance of cumulus cells that surround the developing oocyte, the glucose, lactate and pyruvate levels are adapted to the needs of the cumulus cells. Non-essential amino acids are preferably included in the medium to provide nutrients and avoid subjecting the oocyte to 5 osmotic stress. Essential amino acids and vitamins may also be included to provide nutrients to the cumulus cells. The medium also contains HSA and hyaluronate, which act as a source of macromolecules. Insulin transferin selenium (ITS), insulinlike growth factor (IGF), and epidermal growth factor (EGF) are included to support 10 the function of cumulus cells, which, in turn, nourish and stimulate the oocyte. FSH and hCG are added to stimulate the cumulus and oocyte to undergo changes associated in vivo with ovulation. It should be noted that, when the maturation and medium is prepared, ITS, IGF, EGF and FSH and hCG are preferably the last-added ingredients. The preferred amounts and ranges of these components are found in 15 Table 2.

300 1 10

3. Sperm Preparation and Fertilization Medium

1 7

30

€.0

Current methods of in vitro fertilization employ the same medium for sperm preparation and fertilization as is used for embryo development. No attempt has been made to develop a separate medium for preparation of sperm that is also suitable for storage and support of the oocyte, for promoting the process of fertilization, and for supporting the zygotes formed when fertilization occurs. In many laboratories, the fertilization process is allowed to take place over an extended period which ranges from two to three hours to up to about sixteen (16) to eighteen (18) hours. During this time, the oocyte, sperm, and zygotes produced have significant nutritional needs. In addition, sperm function and fertilization tend to be encouraged when the surrounding fluid contains certain constituents. The sperm preparation and fertilization medium of the present invention is formulated to meet these concerns.

A preferred sperm preparation and fertilization medium in accordance with this invention has virtually the same composition of ions and non-essential amino acids as the oocyte retrieval and handling medium. The fact that these media share a similar ionic and amino acid composition minimizes the stress experienced by the

adopted to the ready of the early the one will be to be to the

oocyte when it is removed from the retrieval and handling medium and placed in sperm preparation medium. Table 3 sets out the preferred amounts and ranges of the ionic and non-essential acid components.

_	Table 3 or ong of matter and of the beginning of
5	Composition of Sperm Preparation and Fertilization Medium*
	Component A the second of the
	Most Preferred PLAZONO WILL BY Preferred Concentration Range
	This is as as the of marked clearly and the content of the content of the content of
	NaCl (100 hours of 100 3.5 - 7.5
	NaH, PO4/2H2O): mainteair a hear that their their than the section of 0.05 - 1.5
10	MgSO4.7H2O Glucose 1 Anni mina co conho suc 201 0.2 - 4.0 0.5 - 5.6
	NaLactate (L-isomer) of a took between distance of Smolintary of the over na habite 2.0 - 20
	NaPyruvate NaHCO3 NaHCO3 NaHCO3 NaHCO3
	स्तर्के स्तर है है असे उन्हें से कहा है कि है है है है है है है से किस है अब नहीं कि से किस है है
15	CaCl2.2H2O 1.8 0.8 - 2.8
	Glutathione 1.0mg/ml 0.5 - 5.0
	Alanine 0.1 0.01 0.01 0.05
20	Asparagine Glutamate 0.1 0.1 0.01 - 0.5
	Glycine is the straight and soften to second on online as distinguished of the of 01016-0.5
	Proline Serine On 1 on 1997 1997
	Serine 0.1 0.01 - 0.5
•	O.O. 1970.0 Hay the tree is a formed with first the entries of the entries of the entries of the entries.
25	HSA programment to the first better the months of the second of the second months different second of the second months different second of the second of th
	The bridge of the state of the
	Penicillin 0.06mg/ml 0.01=10
	Penicillin Streptomycin 0.06mg/ml 0.05mg/ml 0.0110
	addition, spera, truction and fertilization lead to be second and week we cause ading
••	* Concentrations are in millimoles unless otherwise indicated; the medium is aqueous.
30	aqueous. He was a man feel for or he istremed at contraver his sear with
	As will be seen, the sperm preparation medium contains sodium at a higher
	concentration than the level found in the oocyte retrieval and handling medium. This

concentration than the level found in the oocyte retrieval and handling medium. This elevated concentration of sodium promotes sperm function and fertilization, without causing undue osmotic stress to the oocyte. There is also a higher concentration of

25.

phosphate, as compared to the oocyte retrieval and handling medium. The glucose concentration of the sperm preparation and fertilization medium is elevated over that of the oocyte retrieval and handling medium, because glucose is the primary nutrient for sperm and cumulus cells around the egg. The lactate concentration of the present medium is lower than that found in the oocyte retrieval and handling medium, to compensate for the tendency of sperm cells and cumulus cells to give off lactic acid as a waste product. A buffering system is used to maintain the physiological pH, and because sperm preparation and fertilization largely occur within an incubator that can maintain an elevated CO2 atmosphere, a bicarbonate buffer is preferred. Glutathione (not present in the oocyte retrieval and handling medium) is included, to assist in the process of sperm head decondensation. Alanyl-glutamine (present in the oocyte retrieval and handling medium) is omitted from the present medium because it can impair sperin function and reduce fertilization. The same is true of the chelating agent EDTA, which (as will be discussed later) is present in the embryo development media. HSA, the most abundant macromolecule in the Fallopian tube and uterus, is included to support sperm and embryo function. Hyaluronate, which promotes sperm motility, and works in tandem with HSA, is also included. Because sperm tends to contain high levels or bacteria, one or more antibiotic substances are also included. Penicillin, streptomycin, and/or gentamycin are preferred antibiotics. Table 3 sets out the preferred amounts and ranges for these various components.

erapet la <u>the Test Medium</u>a en li l'Alberte, pe di la espera madel el l'Alberte.

5

10

15

20

25

30

In circumstances where it is desired to accomplish fertilization by other than natural interaction of sperm and oocyte, such as where the sperm is unable to fertilize the oocyte due to a thickened zona perfucida surrounding the oocyte, or where the sperm is from a male-factor patient, the sperm may be transported into the oocyte by a technique called intracytoplasmic sperm injection (ICSI). When the ICSI technique is used, the cumulus cells are removed from the oocyte, and sperm is injected into the oocyte's interior using a glass pipette. The present invention contemplates use of a single medium to bathe the oocyte and also to serve as a carrier for the sperm as it is transported by injection into the oocyte. The medium, accordingly, is preferably highly compatible with the interior and exterior of the oocyte. The ionic constituents

To a district account action and the large of the control of the particle of the specific setting

5

10

15

20

25

in the preferred medium are similar to those found in the oocyte retrieval and handling medium, except that phosphate is omitted, to avoid metabolic and homeostatic stress. and the magnesium-to-calcium ratio is 2:1. This ratio of magnesium to calcium is felt to be highly beneficial to the oocyte. Because ICSI is a clinical procedure performed outside the incubator, a buffering system that is effective in a normal atmosphere is an used. MOPS and HEPES are accordingly preferred buffers for this medium. Because the cumulus cells have been removed from the oocyte, and the sperm is at the conclusion of its independent life, glucose, the main energy source for cumulus cells and sperm (but not the oocyte) is omitted from the medium. Pyruyate and lactate levels are increased, as these are a primary energy source for the oocyte. Only the non-essential amino acids most abundant in the oocyte - glycine, proline, serine and taurine - and glutamine (in the stable form alanyl-glutamine) are retained in the ways to medium to avoid osmotic and pH stress and to nourish the pocyte. Preferably, the ICSI medium also includes hyaluronate or polyvinylpyrollidone (PVP), to immobilize or slow the sperm so that they may be captured in the ICSI pipette. Table 4 sets out the preferred amounts and the ranges of these components in the ICSI medium. Moreover, an alternative formulation of the ICSI medium includes hyaluronidase, without which alternative formulation is used to pretreat the oocyte, to break down the hyaluronate gel holding the cumulus cells around the oocyte. This medium is referred to above as denuding medium, and lacks hyaluronate and PVP but includes by the second hyaluronidase. The composition of the denuding medium includes the constituents of the ICSI medium (except hyaluronate and PVP) in the preferred amounts and ranges shown in Table 4 plus hyaluronidase in a preferred about of 40 JU/ml and a preferred range of Oct-80. Optionally, HSA may be included in the denuding medium in the preferred amount of 5mM and the preferred range of 1.0.510mM some of 500 000 onto

33

2 ×

5.3

: ...

31

F. 1

畚

 $\mathcal{D}_{i}^{\bullet}$

syama is from himsle-factor patient, the sports may be can ported into the countries of brights called interacylog farmic aport of ignorion (FOM). When the first modern we nearth, the contribute of is an anomaly from the congregative given is injected in the conjugation order. The product of the constant of the order of the product of the constant of the interaction of the order of the constant of the interaction of the order of the order of the constant of the constant of the order order

De sin es el mombra, sino ménico en alustrios de **Táble 4** de la Carlo de

	Composition of Medium ICSI*
	Compon nt Visited and selection of the Most Preferred Preferred Most Preferred Concentration Range
5	NaCliffy and red month beautiful and dispersion of the 90.08 and the second of the St. of the second
10	MOPS / HEPES 20 10 - 25.0 OF PRICE OF THE P
	NaPyrivate accept at sureig vitating and at an armonic 9.32 and a sureign of 54.0. Out of bases as boseries with early and companies such and a factor of the entire companies of the armonic of the entire of the
15	Alanyl - Glutamine Glycine 9 C 20 = 01 and define the office to a solid cost of 5 and 0.1 - 2.0 Proline Serine Taurine 2004 90 and plants of the cost of 5 and 0.1 - 2.0 Taurine 2004 90 and plants of 5 and 0.1 - 2.0 0.05 - 2.0 0.05 - 2.0
	HSA Smg/ml Hyaluronate of the solution of the
20	* Concentrations are in millimoles unless otherwise indicated: the medium is

* Concentrations are in millimoles unless otherwise indicated; the medium is aqueous.

5. <u>Embryonic Development Medium G1.2.</u>

25

30

The present invention includes an embryonic development medium G1.2. The preferred application of this medium is to support development of the early one-to-eight cell embryo. As depicted in Table 5, the preferred medium has a backbone of ionic constituents and non-essential amino acids that is similar to that found in the oocyte retrieval and handling medium. Unlike the oocyte retrieval and handling medium, the G1.2 medium contains the component EDTA, which supports embryonic development and is believed to bind and disable toxins that might have a deleterious effect on the early embryo, and which also suppresses glycolysis. In addition, this

medium includes HSA and hyaluronate, in concentrations that are thought to support early embryonic development.

5

10

15

20

The preferred formulation of medium G1.2 differs from the previously published medium G1 in several important respects. First, research has shown that an elevated phosphate concentration may not provide optimal conditions for growth of U. the developing embryo. Accordingly, the phosphate concentration has been decreased. Second, hyaluronate has been added to work in tandem with HSA. Third, alanyl-glutamine has been substituted for glutamine. A significant problem for Division embryo culture with amino acids is the natural decomposition of amino acids to ammonium, which decomposition is accelerated at higher temperatures such as the physiological temperature (37 degrees Celsius) used in IVF procedures. Ammonium can be toxic to embryos. Moreover, glutamine is especially prone to decomposition to ammonium within solution. Since embryos are generally cultured in medium G1 or G1.2 for an extended period of up to about 48 hours, a significant quantity of ammonium can develop in the medium and be a significant inhibitor to embryo A. 546. development. Accordingly, the use of alanyl-glutamine provides substantial advantages; it is a particularly stable form of glutamine and is not prone to breaking down in solution. Also, the concentration of alanyl-glutamine in G1.2 has been $s \mathbb{R} A$ reduced to .5 mM. These three modifications make G1.2 a significantly improved in the control of medium for early embryonic development over medium G1. The most preferred amounts and preferred ranges of the components of medium G1.2 are depicted in Table 5. Les militares consophaire mendro esche entomalier ai me more passence. Sijusous.

Subryonic Developer can Make and CD. 2.

F. . . .

٤.٠

: :

3

The present invasided includes an antiquarin development of the conjugate the present development of the newtons is to appear development of the conjugate of the newtons is to appear development of the conjugate of the newtons is to appear the conjugate of the

	·	•			70377/20408
	of the property of the	mudically in a superior	Table 5		
		Composi	tion of Medium G 1	<u>.2</u> *	
	Component		. 11 A C L A L		, B
		uminater em hava el a c			Preferred
				• • •	
	ं ्रे भितासीधा	a ni fiprapiani en l'existino	TO SHOW TO THE SECTION	\$ - S - S - S - S - S - S - S - S - S -	Range
	NaCl	र अध्यक्ति देवी देव देखालाहरू । इस्त	90.08	- 15.	90.0 100
5	KCl		5.5	* 747 .	80.0 - 100
	NaH,PO,2H,O	and Jacob Children	0.25	ा ५ प्रकृत	3.5 - 7.5
	MgSO ₄ .7H ₂ O -	nero do gali dom e da d	0.23		0.05 - 1.5
	Name ()		2.5		
	के ती नवाद वर्तते	advant et par bereint	13 1 1 1 2 1 3 1 1 1 1 1 1 1 1 1 1 1 1 1		15.0 - 30
	CaCl ₂ 2H ₂ O		1.0		
		क्षाक दूरा र देवी विकास स्थान	, 11, 31 - 32 / 11.8 - 1 - 32 - 32 -		0.8 - 2.8
10	Glucose	ม และการ สม โดกสมโดก สมเด	មានដល់ន ្តីទ ួល នេះស		
= =					
		L-isomer) Approved the			
	i barniant and C	our penerts of modities of 2.	0.32 ar/ gamagaire	al a contrator a pr	0.1 - 1.0
	Alanine	· · · · · · · · · · · · · · · · · · ·			
	Asparate		0.1	•	0.01 - 0.5
15	Asparagine		0.1		0.01 - 0.5
15	Glutamate		0.1		0.01 - 0.5
		ತ <i>್ತಿಕೆ</i> ಕ್	0.1		0.01 - 0.5
	Alanyl - Glutami	ne <u>1995 (1971)</u> 19	0.5		0.1 - 1.0
	Glycine Proline		0.1		0.01 - 0.5
20		-	0.1	<u>-:</u>	0.01 - 0.5
2011	Serine		0.1		0.01 - 0.5
# <u>%</u>	Taurine	g topic gas	0.1	•	0.01 - 10.0
·	. ,	**			
1921 2	EDTA		0.00		
		€0.0°	0.01	i i	0.005 - 0.20
		5.5			
	- 20:0 uc a	₹ 0		1. 3. 1. 3.	1317
	HSA		5mg/ml	TAPP 4 Ta	1 - 10.0
=	, Hyaluronate	2.3	0.1 mg/ml	(0.1	0.02 - 0.5
	* 0	5 I		62711	Car ()
ري 23	· Concentrations a	are in millimoles unless of	herwise indicated; th	ne medium isser.	127 T 3
	caqueous.	787	į «	1980 11 JA 2011 128	عد. ا
	- M.C			32.% tss	$q_{m,i} = 1$
		bryonic Development Med	<u>dium G2.2</u>	- ÷ ::	.``
	Medium G	2.2 is also formulated to su	upport embryonic de	velonment Ita	, . ·
	- 10.0		apport cinoryome de	sverobinetiť ils	4. B
< € ·	preferred use is wi	th embryos from the eight	-cell to the blastocys	t stage (around l	00
30 ^{0.0}	cells) to around on	e-hundred cell stage. The	backbone of ionic of	onstituents and -	esty of
3.11 (1.4)		ds preferably found in me			
į.	with medium G1 2	event that the		any me same as	usea ;;
		, except that the concentra			
253	increased. This red	luces the risk of subjecting	the embryo to osme	otic stress as it is	
		· · · · · · · · · · · · · · · · · · ·		one sucsses it is	

PCT/US99/28408 WO 00/32140

moved from medium G1.2 to medium G2.2. Taurine is omitted because its benefits to the embryo appear to be confined to the period prior to compaction. Glucose, lactate and pyruvate are included as carbohydrates, except that the concentration of glucose is increased, while lactate and pyruvate are decreased, as compared to medium G1.2.

- This modification in carbohydrate levels is in response to the increasing ability of the developing embryo to metabolize glucose as an energy source, and reflects also the observed composition of the female reproductive tract. Eagle's essential amino acids are included in medium G2.2 because they are necessary to stimulate the growth of the inner-cell mass of the blastocyst. Vitamins are added as a group because in the inner-cell mass of the blastocyst.
- animal studies they tend to facilitate the function of the blastocyst, including fluid accumulation in the cavity of the blastocyst. Importantly, this medium lacks EDTA. The preferred amounts and ranges of the components of medium G2.2 are depicted in Table 6.

观点 人名英德

3,0000

Table 6

St. 72 11 1

ं ग्रेक्टक प्राप्त

21/1 Hat 1 F

Composition of Medium G 2.2*
- TT () () () () () () () () ()
Component A Stiffer B
Most Preferred Preferr
Concentration Rang
NaCl 90.08 480:0 - 1
KCl 5.5 3.5 - 7
NaH2PO4.2H2O 0.25 0.05 - 1
20 01 MgSO4.7H2O halvane 1 /0.2 - 4
NaHCO3 16 Na. 1.3 25 25 25 25 25 26 27 215 25 20
C ₂ C ₁₂ 2H ₂ O 1.8 0.8 - 2
Glucoses in choises she ben alive a comedia and 3.15 or thin alives excite the co. 0.5 - 5
NaLactate (L-isomer) 5.87
25 NaPyruvate 0.1 0.01 - 1
Alanine (2) and the second of
Asparate 0.1 - 0.01 - 0.01 - 0.01
Asparate Asparagine Only the second of the
- $ -$
30 Alanyl - Glutamine 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
0.01 - C
Proling the set of a total address to the configuration of Q.1 The first of 2500 Configuration (#01 - C
Serine 0.1 - 0.01 - 0
Serine L-Arginine-HCl 0.1 0.1 0.1 0.1 0.1-1
35 L-Cystine 2HCl., we wond to a real to got of the 0.1 a seine descended of the 100:05 = 0.

			PCT/US99/28408
L-Histidine-HCl-H2O	•	0.2	0.1 - 0.4
L-Isoleucine	2° ;	0.4	0.1 - 0.4
L-Leucine		0.4	
L-Lysine-HCl	* *	0.4	0.1 - 0.8
5 A-Methionine			0.1 - 0.8
L-Phenylalanine	3.10	0.1	0.05 - 0.25
	 2	0.2	··· 0.1 - 0.4
L-Threonine	18.5	0.4	0.1 - 0.8
L-Tryptophan	<u> </u>	0.5	7 0.1 - 0.9
L-Tyrosine 2Na	:.	0.2	0.1 - 0.4
10 / L-Valine	والجوار والمتار	0.4	0.1 - 0.8
D-Ca Pantothenate	$^{r}\mathcal{K}$	0.002	- 0.001 - 0.004
Choline Chloride	?	0.007	0.003 - 0.01
Car Folic Acid	Ī	0.0023	9-75-7 (0.001 - 0.0045
i-Inositol	1.4	0.0111	0.005 - 0.02
15 V Niacinamide		0.0082	0.004 - 0.016
🐫 - Pyridoxal HCl	1.6	0.0049	0.002 - 0.01
Riboflavin).ć	0.0003	0.002 - 0.01
Thiamine HCl	1 (0.003	0.0001 - 0.000
· · · HSA	en g San		0.001 - 0.006
20 Hyaluronate	4	5mg/ml	1 - 10.0
20 1 1 Salutionale	14	0.1mg/ml	ST HO 0.02 - 0.5
2.0 3 1			6 to 18 in 19

DCT/I ICOO MO 400

273.3.

3 4 3. 1.

.2

 $\tilde{\boldsymbol{\beta}} \notin$

ij....

aqueous. 6.1 - 3.3

3 C = 1.0 0.1 - 0.4

91-23

35

WO 00/32140

7. **Embryo Transfer Medium**

The preferred embryo transfer medium contains the same formulation of

25 constituents as medium G2.2 except that a much higher concentration of hyaluronate CENTA - 14 0.0 is included. In the human reproductive system, research indicates that there is a receptor on the embryo for hyaluronate and that there is also a receptor for the control of the hyalurcnate on the endometrium of the mother. Hyaluronate is thought to act like a

biological glue that assists the embryo in binding to the endometrium and, which care accordingly, supports implantation. The preferred amount and ranges of the same in the preferred amount and the same in the preferred amount and the same in the constituents of the embryo transfer medium are depicted in Table 7.

Table 7 Composition of Embryo Transfer Medium*

the addition of the following of the dependency of the continuence of

Component	Most Preferred	<u>B</u>
Figure with the	Most Preferred	Preferred
	Concentration of the concentration	
NaCl Hawa	ं पूर करता क्षणात्रात्र संस्थाति है। एक सिन्स तु स्थापी है कार्यमार्ग है। इस स्थाप	80.0 - 100

^{*} Concentrations are in millimoles unless otherwise indicated; the medium is

WO 00/32140	-PCT/US99/28408

ſ.	KCl	S .G	5.5	(189-8 Malemalia : 3.5 - 7.5
	NaH2PO4.2H2O		0.25	ുത്യ ക്രമ 0.05 - 1.5
2 G.	MgSO4.7H2O	±.0	1	es 1960 0.2 - 4.0
,	NaHCO3	54 CF	25	1999 may 45 - 30.0
5	CaCl2.2H2O	t.S	1.8	naturolicite#d 0.8 - 2.8 ù
. 1	Glucose	3.0	3.15	96 v kilýzmy2 0.5 - 5.5
. :	NaLactate (L-isomer)	•	5.87	<i>૱ઌઌ</i> ૹઌ ૼ2.0 − 20.0
	NaPyruvate		0.1	. weighting (0.01 - 1.0).
p .	Alanine		0.1	EVES # 1672 TY 0.01 - 0.5
10	Asparate	, O.	0.1	0.01 - 0.51
20 G	Asparagine	5.70	0.1	erson Conset (10.01 - 0.5
	Glutamate	* () () ()	0.1	4.7 × 30 5 × 0.01 - 0.5
	Alanyl - Glutamine		1	##58 J.0.01 - 2.0
	Glycine	37133	0.1	. 1995 o o o o o o o o o o o o o o o o o o
15	Proline	\$200.40	0.1	#80granic 0;01 - 0.5 €1 -
3	Serine	0,000	0.1	(CV) to sub (Q.04 - 0.5
- 13 - 11 -	L-Arginine-HCl	£1 (#2 c	0.6	marsho@4 - 1.2
	L-Cystine 2HCl	2 1 K 1 6	0.1	10 No. 10 No. 10 10 10 10 10 10 10 10 10 10 10 10 10
. 1	L-Histidine-HCl-H2O	Jan Street Bridge	0.2	0.1 - 0.4
20	L-Isoleucine	12 g. 10	0.4	23 10 1 0.1 - 0.8 115
•	L-Leucine		0.4	0.1 - 0.8
	L-Lysine-HCl	San	0.4	0.1 - 0.8
	L-Methionine	(2) 1 (1) (1) (2) (2) (2) (2) (2) (2) (2) (2) (2) (2		that if we enough to 0.05 - 0.25
	L-Phenylalanine		0.2	0.1 مر 0.4
25	L-Threonine		0.4	0.1 - 0.8
	L-Tryptophan		0.5	0.1 - 0.9
	L-Tyrosine 2Na			0.1 - 0.4
	L-Valine		0.4	0.1 - 0.8
	D-Ca Pantothenate	The state of the state of	0.002	(746° 5 hard larger 0.001 - 0.004
30	Choline Chloride	्रात् विकास स्टिबंदित	2 20.70.00 to 10.00.00 to 10.00	27 10.0 5,809.0 state as medium G2
	T 1' A ' 1		41 614 1 7 4	0.001 - 0.0045 o narrani ed sa b. 0.005 - 0.02
	i-Inositol	ACTE TO SECTION OF SECTION	0.0111	0.003 - 0.02
	Niacinamide (Antique)	3 11 8 8 8 CC 13	/1 5.m. 0.00821EV	0.004 - 0.016 on the appropriate left 0.002 - 0.01
	Pyridoxal HCl		0.0049	0.002 = 0.01 0.0006 = 0.00000
35				
	Thiamine HClaus 125 and 125	ສາວວິດຕາສຄົນ ອະວຸດທັ	raft, at 0,000 are of	600.0 5.100.00 give tractessists of
	Hyaluronate The Spect	ton in classica	0.25mg/mi	udema stroegas vitari 0:050e1.0
		da Ting hot web a	a alajajorent istest	व्यवसायिक का विव्यवस्था है।
:	*Concentrations are in m	illimoles, unless	otherwise indicate	ed; the medium is

aqueous.

Cryopreservation Medium and he cost a country 8.

40

The present invention involves a separate medium to be used in cryopreservation of the oocyte and embryo. The preferred formulation to be used includes ionic constituents and a buffer, preferably MOPS or HEPES, as well as the $\frac{10.5 \, \mathrm{M}_{\odot}}{10.5 \, \mathrm{M}_{\odot}}$

? }

carbohydrates lactate, pyruvate and glucose. Optionally, HSA may be included. In addition, the medium may include certain additives, glycerol, ethylene glycol, DMSO, propanedial, and/or sucrose. The preferred amounts and ranges of the constituents of the cryopreservation medium are depicted in Table 8

	की प्रकार पुरस्कार प्रकार स्थापन क्षेत्रकार कार्य, वास्तु वा कार्या वा वा वा वा प्रकार प्रकार प्रकार कार्या
5	Table 8
	good of the control Composition of Cryopreservation Medium* and the new control of the control o
	Components it can be a substantial asymmetric for the $oldsymbol{A}$ the second $oldsymbol{\mathbb{Z}}$, where $oldsymbol{\mathbb{Z}}$
	Most Preferred Preferred Preferred Concentration
	NaCi 90.08
	cose gratation decision principals of his alternation and tyle or figure 2011. The first is
10	1 Substitution accorded with the most built of seeming of the difference of the content of the MgSO ₄ , 7H ₂ O
	ນ ໃຊ້ເປັນການເປັນເປັນໄດ້ກ່າວ ຂອງເປັນ ພາກເປັນເປັນການຕົວເປັນ ກາວແລະ ໃຫຍ່ເປັນຄົວເປັນ ຄົວເປັນ ການ 1997 - 4
	Na2PO4.2H2O
	the stitute this property of a property of the contraction of the supposition and
	NaHCOs neiw some collaboration of the first of 5 to 10 to 10 to 20 - 10
	MORS/HERES: ANDVIOLENCE BY LONG TO SEE 20, Let 10 - 25.0
	க ை VI நணி சிற்கும். என்ற குண்கும் முழு இரை படர்கிற படர்களின் படர்களின் க
	CaCl ₂ .2H ₂ O 1
15	NaLactate (L-isomer) 3.51.57 1 5.87 2.0 - 20
	NaPyruvate 11 best in the ed at such bong share a 0.32 2 miles
	Glucose 1,000 si 21/17 Agreeme situation a seri an ri. (20) pla con a montro contact agreement in the contact agreement i
	e en financial en la la colonia de la colonia de la colonia de la colonia de manos volumente en la colonia de l
	ourse. It has a suidat so de Touri ou house us a farsaire a la sale artour house a la
20	ADDITIVES of operious of Tours to the part was a second of the part of the par
20	Glycerol and/or ethylene glycol and/or DMSO and/or propanedial and/or sucrose Range for all except sucrose is 2 to 20%; range for sucrose is 0.1 to 1M
	Concentrations are in millimeters unless otherwise indicated; the medium is aqueous
	Allstoods a quita formachi arla de mante a les anno longit en les anno de la company d
	B. Sequential Culture Media Process and France and the control of the extension
	Instead of immersing human reproductive cells in a single culture medium
25	throughout the various procedures used in IVF, the present invention involves a
	2 Coling in Action in Acti

process by which the reproductive cells may be moved through a sequence of distinct culture media as the various IVE procedures are carried out. In one aspect of the invention, the culture media are specifically formulated to provide a physical in sequence environment similar to that found within the female reproductive tract and conducive to growth and development of human reproductive cells during various stages of the IVF process. In a further aspect of the invention, the specifically formulated culture media can be applied to support the reproductive cells in one or more of the following procedures: oocyte retrieval and handling; oocyte maturation; ordinary fertilization; oocyte, zygote and embryo examination and biopsy; embryonic development to the eight-cell stage; embryonic development to the blastocyst stage; embryo transfer; and cryopreservation. Most preferably, the media will be applied sequentially during each of the applicable stages of the IVF process to which the media have been adapted. It should be noted that there is significant variation among clinics and laboratories as to equipment and specific procedures used to accomplish each of the principal steps in the IVF process. The present invention contemplates that the sequential media and process described herein may be utilized and/or readily adapted for use with the wide variety of equipment and procedures employed in IVF practice. What follows is a second more detailed discussion of exemplary applications of the media during IVF and Calls 2B,O c related methodology:

94

() \$

73.

5

10

15

20

25

30

1. Oocyte Retrieval and Handling; Embryo Handling

Referring to Fig. 1, an initial procedure in the illustrated IVF process 100 is oocyte removal or retrieval (102) from the mother's ovary. This is typically performed vaginally using a fine needle attached to and guided by a transvaginal ultrasound probe. The needle is ordinarily connected to fine Teflon tubing and thence to an aspiration regulator controlled by a vacuum regulator. The aspirate is collected in test tubes or other appropriate vessels, containing medium. The medium may be used to preliminarily wash the needle and tubing, and other equipment used in the procedure. In some clinical settings, the medium may also be used with a specially adapted needle to flush the follicle and aid in removal of the oocyte. The medium, equipment used, and aspirate are maintained, so far as possible, at 37 degrees Celsius. If a bicarbonate buffer system is used in the medium, the procedure ordinarily is

^{21 2} WO 00/32140 PCT/US99/28408

carried out in a gassed humidicrib or isolette which maintains a 3%-10% CO₂ atmosphere. In the absence of such atmospheric controls, the medium must contain a MOPS or HEPES buffering system.

The illustrated process 100 present invention contemplates that the oocyte retrieval and handling medium may be used in each phase of the retrieval process. The process of using the oocyte retrieval and handling medium may involve washing any equipment that may come into contact with the oocyte during removal from the ovary, and that may be used to aspirate, flush and/or wash the oocyte during the removal and collection process. Following removal from the ovary, the oocyte may be washed with medium. Optionally, the oocyte may be stored in the medium for a period:

1

 \mathfrak{J}^{r}

In addition, it is contemplated that the medium may be used during other clinical or laboratory procedures where the oocyte is manipulated or handled, and also in procedures where the emoryo is manipulated or handled, especially where these occur outside the isolette. Examples would include examination of the oocyte following retrieval from the mother, examination of the oocyte following the fertilization step, and examination of the embryo to determine whether it has developed the eight-cell stage. In each of these examples, the oocyte/embryo would be bathled in the medium as it is withdrawn by pipette from the culture dish or test tube, and would remain immersed in the medium while examined under a microscope or with other equipment. The illustrated implementation of the invention also contemplates that an alternative formulation of this medium, which is calcium and magnesium free, may be used during biopsy procedures.

a the back releasting her relies as your resembles in each case or named in

2. Occyte Maturation of the trouble of the transfer of the Art and

5

10

15

20

25

30

envisions that a second medium may be used to support and promote development of the oocyte during maturation (106). The oocyte maturation medium would ordinarily be used to treat and mature the oocyte following a collection procedure, in which the oocyte is retrieved from the ovary using oocyte retrieval and handling medium. The retrieval and handling medium and maturation medium have a very similar backbone of ionic constituents and amino acids and glutamine, such that as the oocyte is moved

process 100 includes immersing the oocyte and surrounding cumulus cells in the maturation medium for a period of about 30-48 hours, or until the oocyte is mature. The illustrated process 100 then contemplates removing the oocyte from the maturation medium and immersing it in either sperm preparation and fertilization medium or ICSI medium for purposes of fertilization.

In accordance with the invention, the oocyte maturation medium may be applied to the oocyte retrieval process (102), in place of the oocyte retrieval and handling medium described herein. Additionally, a conventional culture medium, such as Ham's F-10 or medium TCM-199 with or without a HEPES buffer, may be employed for immature oocyte retrieval and handling, before immersion of the oocyte in the maturation medium of the present invention. Once maturation is complete, the oocyte will be immersed in a medium for ordinary IVF fertilization procedure (110), or will be immersed in an ICSI medium in preparation for assisted insemination through an ICSI procedure (112).

(3.,

έŅ

3. Sperm Preparation and Fertilization and Section of the section

5

10

15

20

25

30

The illustrated process 100 contemplates that the collected oocytes will ordinarily be washed and immersed in, and allowed a period of pre-incubation culture within, a first portion of the sperm preparation and fertilization medium. This period of pre-incubation culture (104) may last up to about six (6) hours. Oocytes permitted a period of pre-incubation culture typically have higher fertilization rates.

as your anacheodracal teventer grind and a

The process 100 also contemplates that the sperm may be separately washed and stored in a second portion of the sperm preparation and fertilization medium to purge it of bacteria and any other contaminants that may be prosent. Sperm preparation (108) may involve dilution of semen with the medium, centrifugation, and resuspension of the concentrated sperm in a new portion of medium. In the "swim up" method of sperm preparation, the medium containing sperm is centrifuged, the medium is drained off, and a new portion of medium is poured over the spundown sperm pellet. The sperm is given a period to "swim up" into the fresh medium. That layer of fresh medium, containing the more motile sperm, is then poured off and centrifuged, and the process is repeated. In another aspect of the invention, the sperm

preparation and fertilization medium may be used in one or more gradient separation procedures, such as the Percoll procedure. The present invention envisions that the sperm preparation and fertilization medium may be used as the medium in any of the sperm preparation procedures that may be used for IVF.

5

10

15

20

25

30

Once the sperm is prepared (108), the sperm is then examined and counted while in medium, and a desired quantity is added to the portion of medium which contains the oocyte. The sperm and oocyte are permitted to remain together in the medium for a period of up to several hours, and, in some laboratories, for a much longer period, as long as about sixteen (16) to eighteen (18) hours. The invention further contemplates that, following a period of immersion in the medium with sperm, the oocytes will be removed and examined (114) to determine whether fertilization (110) has occurred. When removed for examination, the oocytes will continue to be bathed in the sperm preparation and fertilization medium if the examination can be conducted in an isolette. If not, then, as noted above, the oocyte retrieval and handling medium may be used for handling and examination of the oocytes.

Technique) orienti appropriation to the Ocyte (ICSI) or in

Albert of Head Officer and James Are to be

In the ICSI process (112), sperm may be directly injected into the cytoplasm of the oocyte through a very fine pipette or needle. The process 100 contemplates washing the sperm with a portion of the ICSI medium containing hyaluronate and/or PVP, and then placing the sperm in the medium. The process 100 further involves drawing a microvolume of the medium containing sperm into the pipette and then injecting the medium and sperm into the interior of the oocyte.

Ĩ.;

The illustrated process 100 further contemplates that the oocyte may be bathed in another portion of the ICSI medium during the ICSI process. An alternative formulation of the ICSI medium may be used, supplemented with hyaluronidase, for denuding pretreatment (105) of the occyte prior to the ICSI process. Pretreatment involves immersing the oocyte in the ICSI medium supplemented with hyaluronidase for a period until the oocyte becomes denuded of all or most of its surrounding cumulus cells. Following pretreatment, the occyte is injected with sperm carried in a separate portion of medium, using an ICSI pipette, as provided above.

After the ICSI injection process (112) is complete, it may be necessary to examine (114) the occyte to evaluate whether fertilization has been effective and the occyte is intact and healthy. Examination may occur in the ICSI medium bathing the occyte, or may occur in the occyte retrieval and handling medium as described above.

13 5. Embryonic Development to Eight-Cell Stage by but, and the property of the Eight-Cell Stage by the but, and the property of the Eight-Cell Stage by the but, and the property of the Eight-Cell Stage by the but, and the property of the Eight-Cell Stage by the but, and the property of the Eight-Cell Stage by the Ei

5

10

15

20

25

Medium G1.2 is applied to the early embryo, following formation of the zygote. After the zygote is identified, it is washed with medium G1.2, and then immersed in G1.2 medium for a culturing period (116) of up to about forty-eight hours. During this time the embryo undergoes development from the one-cell to around the eight-cell stage, and is removed at about the eight-cell stage. Examination (118) of the embryo may occur in the G1.2 medium, or in the obcyte retrieval and handling medium, as described above.

the and are income in the second of the first and a second of the second

 $\mathcal{L}^{\mathcal{T}_{pk}}$

6. Embryonic:Development to Blastocyst Stage and term in the transfer and the second s

The illustrated process 100 contemplates that medium G2.2 will be used to culture (120) the developing embryo to the blastocyst stage, preferably from about the eight-cell stage to about the one-hundred-cell stage. The process 100 also contemplates that, once the embryo reaches the blastocyst stage, and assuming that the embryo is judged on examination (124) to be viable, it is removed from the G2.2 medium and prepared for transfer into the uterus. In some laboratories, the G2.2 medium may, optionally, be used for embryo transfer as well. Examination (124) of the embryo may occur in the G2.2 medium or in the occyte retrieval and handling medium, as described above.

7.0-31 Embryo Transfer of 1805 and gain all conflicts (201 and 10 notines, confidence of

The process 100 contemplates that the embryo transfer medium will serve as a carrier for the embryo as it is transferred (126) back into the mother. The embryo will be bathed in the transfer medium, the medium containing the embryo will be drawn into the transfer catheter, the catheter will be inserted into the mother's uterus, guided by an ultrasound probe, and the medium containing the embryo will be injected into the uterus.

5

 \cdot \cdot

· / .

8. <u>Cryopreservation</u>

The cryopreservation medium may be used for storing, freezing, thawing, vitrification, and warming the oocyte, prior to fertilization. The same medium may be used for storing, freezing, thawing, vitrification, and warming the cleavage stage embryo, as well as the embryo in the eight to one hundred cell stage.

While the present invention has been described in relation to one embodiment, it will be appreciated that the invention may be utilized in numerous additional embodiments and procedures. Such additional embodiments and procedures are within the scope of the present invention, as defined by the claims which follow.

Treappare on the brakes of these for the configuration of the last of the configuration of the last of

の jie tu tela fin et ner en gese finot a fin en fin (人) in the internation of energy eight telepolities (人) in the internation of engage to

Be now a little of the second of the second

अर्थनेत्रात त्रिक्ष्यक स्थानी वेत्रास का कार्या के स्थान है। अर्थन का अर्थन विकास अस्त्राहरू विकास विकास का अर उस्तर सामान्य सामान्य कार्या व्यापन कर्मा व्यापन कर्मा कर्मा व्यापन कर्मा कर्मा सामान्य कर्मा कर्मा कर्मा कर्म

The state of the s

महाराज्याम सहस्कृतक महाने जिल्ला का उत्तर हो । अस्तर १००० विकार महाने कि स्वार का कार्य का विकार का जाना है ।

od serfisse i modyddeson. Gell ei genegol e'i ei eendd fol ei alw 3, e boeres add rool swygodd earliadd.

ិស្ស គ្រង់ គ្រង់ ម៉ាស្រីស្នងសម្រង់ និង និង និង និង ម៉ាស់ស្រីស្នងសម

THE PROPERTY OF THE PROPERTY OF THE CONTRACT OF THE PROPERTY O

and description and decreptions of the Control of t

CO. BLANDS.T.

5

10

15

20

25

What is claimed is:

1. A method for use in an IVF process, wherein the process involves some or all of the stages of: oocyte retrieval and handling; oocyte maturation; sperm preparation; fertilization; oocyte, żygote and embryo examination and biopsy; embryo development; embryo transfer; and cryopreservation said method comprising the steps of:

supporting reproductive cells in a first support medium during a first stage of said stages, said first support medium including a core group of salts; and

first support medium during a second stage of said stages, said second support medium including substantially said same core group of salts as said first support medium, said core group of salts utilized in both of said first and second support media thereby minimizing any stress and trauma to reproductive cells incident to transfer between the first and second support media;

wherein no more than one of said first and second stages is one of said embryo development stage and said embryo transfer stage.

- 2. A method as set forth in Claim 1, wherein said first stage is one of embryo examination and oocyte retrieval and handling.
- 3. A method as set forth in Claim 2, wherein said first support medium comprises water, ionic constituents and a buffer.
- 4. A method as set forth in Claim 2, wherein said first support medium comprises one of 4-Morpholinepropanesulfonic acid (MOPS), N-2-hydroxyethylpiperazine-N¹-2-ethane sulphonic acid (HEPES) or bicarbonate.
- 5. A method as set forth in Claim 2, wherein said first support medium comprises carbohydrates.
 - 6. A method as set forth in Claim 2, wherein said first support medium comprises non-essential amino acids.
 - 7. A method as set forth in Claim 2, wherein said first support medium comprises glutamine.
- 8. A method as set forth in Claim 2, wherein said first support medium comprises antibiotics.

٠,,

્ •્

⊎# WO /00/32140	PCT/US99/2
at it: 9 A method as set forth in Cla	im 1, wherein said first support medium is
free from calcium and magnesium and said	
procedures.	
10. A method as set forth in Clai	m 1, wherein said first stage comprises
oocyte maturation.	_
11. A method as set forth in Clai	m 10, wherein said step of supporting
reproductive cells in a first support medium	
first supportimedium for a time period follow	
development prior to fertilization.	માં મુ લ
of a gar 12 has a A-method as set forth in Clair	n 10, wherein said first support medium
	• • • • • • • • • • • • • • • • • • • •

15.

5

10

15

20

25

30

comprises magnesium and calcium disbursed in an aqueous solution.

13. A rnethod as set forth in Claim 10, wherein said first support medium comprises one or more of non-essential amino acids, essential amino acids, cysteamine, human serum albumin (HSA), and hyaluronate.

14. 14. A method as set forth in Claim 10, wherein said first support medium comprises one or more growth factors such as insulin transferin selenium (ITS), insulin-like growth factor (IGF), and epidermal growth factor (EGF).

SHOW 15: 1 WA method as set forth in Claim 10, wherein said first support medium comprises one or more hormones such as follicle stimulating hormone (FSH) and human chorionic gonadotrophin (hCG). Which will be a seed to be a

15016. 150 Amethoa ac set forth in Claim 1, wherein said first stage comprises 300 one of sperm preparation and fertilization.

- A method as set forth in Claim 16, wherein said first support medium comprises carbolity trates place a refer of a median in a refer a contract of a
- A method as set forth in Claim 16, wherein said first support medium 18. comprises one or more of bisarbonate, glutathione, HSA and hyaluronate.
- A method as set forth in Claim 16, wherein said first support medium comprises antibiotics: The same country as a facility of the case the
- A method as set forth in Claim 16, wherein said first support medium 20. comprises nonessential amino acids. A all p by D do a rest of the base
- 21. A method as set forth in Claim 16, wherein said first support medium 35 Philippe Williams and a is free of EDTA.

177

5

10

15

20

25

30

<i>i :</i>	22.	A method as set for	orth in Clain	11, wher	ein said firs	t stage comprises
оосу	te retri	ieval and handling and	said second	l stage co	omprises on	e of sperm
prepa	ration	and fertilization.				FOR THURST!

- 23. A method as set forth in Claim 22 wherein said second support medium has an elevated concentration of sodium as compared to said first support medium.
- 24. A method as set forth in Claim 22, wherein said second support medium has an elevated concentration of phosphate as compared to said first support medium.
- 25. A method as set forth in Claim 1, wherein said first stage utilizing said first support medium is part of a process of intracytoplasmic sperm injection (ICSI)....
- 26. A method as set forth in Claim 25, wherein said ICSI process? comprises removing cumulus cells from an occyte, incubating sperm, and injecting the sperm into said oocyte; and toy and (1908) method is manes contained.

said method further comprises the step of placing the sperm injected oocyte into said second grants for the land of the said second grants for the land of the said second grants for the land of the said second second grants for the land of the said second second grants for the land of the said second s

- used in said-ICSI process is free from phosphate.
- 28. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises one of MOPS process, HEPES and bicarbonate.
- 30. A method as set forth in Claim 25, wherein said first support medium as used in said ICSI process is free of glucose. A contract A as horizon A
- 31. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises non-essential amino acids.
- 32. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises glutamine.
- 33. A method as set forth in Claim 26, wherein said first support medium is used for supporting said sperm as part of said ICSI process and comprises one of hyaluronate or polyvinylpyrolidone (PVP).

34: A method as set forth in Claim 25; wherein said first support medium comprises magnesium and calcium in an aqueous solution.

- A method as set forth in Claim 25, wherein said first stage comprises denuding an oocyte and said first support medium comprises hyaluronidase.
- and 36.00 A.method as set forth in Claim 1, wherein said first stage comprises embryonic development.

5

10

15

20

25

30

- reproductive cells in a first support medium comprises supporting a zygote in said first support medium for a time period that is one of at least 48 hours or through at least the eight-cell stage.
- comprises carbohydrates.
- 39. 76 A method as set forth in Claim 36, wherein said first support medium comprises non-essential amino acids.
- 40. A method as set forth in Claim 36, wherein said first support medium comprises one or more of HSA, and hyaluronate.
 - Amethod as set forth in Claim 36, wherein said first support medium comprises glutamine.

 - supporting reproductive cells in a third support medium different than said first and second support in Junis during a third stage of said stages.
- 44. A method as set forth in Claim 43, wherein both said second stage and said third stage comprise embryo development and transfer.
- 45.22 A method as ser forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has a depressed concentration of one of lactate and pyruvate relative to said second medium.
- 46. A method as set forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has an elevated concentration of glucose relative to said second support medium.

WO 00/32140	(::- PCT/US99/28408
47. A method as set forth	nin Claim 36, wherein said step of supporting
reproductive cells in a first support r	medium comprises supporting an embryo in said
first support medium for a time period	od that is one of from about 48 to 96 hours and
from about the eight-cell stage to ab	out the one hundred cell stage. See the tribback
48. A method as set forth	in Claim 36, wherein said first support medium
comprises non-essential amino acids	s and is free from taurine. Inserting the teach place of the country
49. A method as set forth	in Claim:36, wherein said first support medium
comprises essential amino acids.	्रात्तिकारी कारतींड कि व्यक्तिया है
50. A method as set forth	in, Claim, 36, wherein said first support medium:
comprises vitamins.	September of the second state of the second state of the second s
51. A method as set forth	in Claim 36, wherein said first support medium
comprises HSA.	comprises corbohycratics.
52. A method as set forth	in Claim 36, wherein said first support medium
is free from EDTA.	elither actions between the entire
53. A method as set forth	in Claim 36, wherein hyaluronate is added to
	o transfer. ে বিভারত, ১৪৮৭ সিলেল কে তেও কলে কলে কলে কলে কলে
54. A method as set forth	in Claim 1, wherein said first stage comprises
cryopreservation.	.គេកា ភូពខេត្តខ្លាំង នេះ បាក់ការ
55. A method as set forth	in Claim 54, wherein said first support medium
comprises one of MOPS or HEPES.	Joay Lynn mine.
56. A method as set forth	in Claim 54, wherein said first support medium
****	supporting reproductive cells in a third or per a read
57. A method as set forth	in Claim 54, wherein said first support medium
	र्यः ते त्राव्यक्तियो १८ वर्षा के तो के विकास में अपन
	in Claim 54, wherein said first support medium has
• •	thylene glycol, DMSQ, proparediol/and sucrose.
59. A method as set forth	in Claim 36, wherein said first support medium at 2

5

10

15

20

25

30

comprises EDTA.

60. A method as set forth in Claim 54, wherein said first support medium horn comprises nonessential amino acids, and the first support to the body on A 104.

and the state of t

1.5

3 £

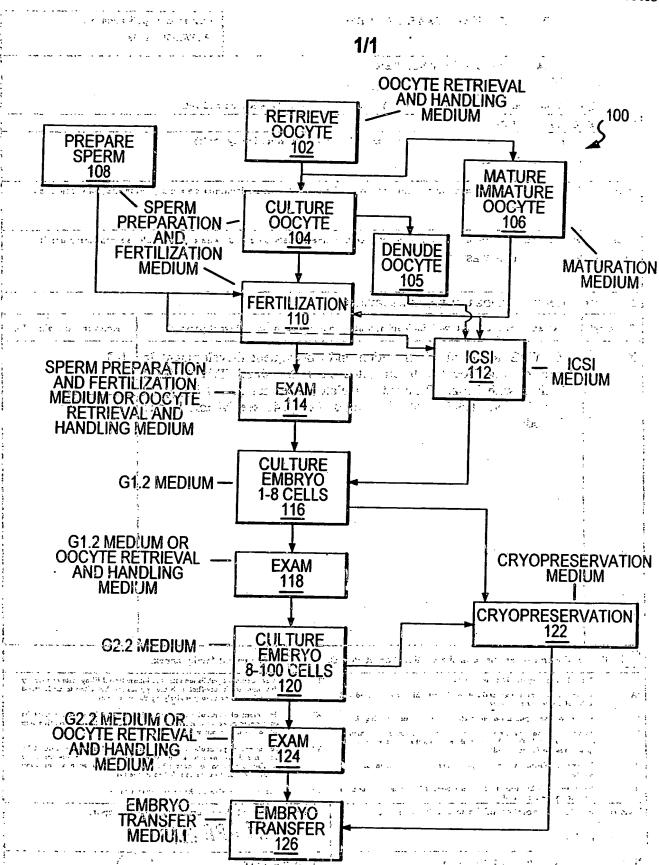


FIG 1

INTERNATIONAL SEARCH REPORT

International application No. PCT/US99/28408

"知识是话。""

A. CLASSIFICATION OF SUBJECT MATTER IPC(7) :A61F 5/58; A01N 1/02	
US CL :435/2; 600/23, 24, 25 According to International Patent Classification (IPC) or to bot	sk material of Difference and IDC
	in national classification and IPC
B. FIELDS SEARCHED	
Minimum documentation searched (classification system follow	
U.S. : 435/2; 600/23; 24, 25	The second secon
Documentation searched other than minimum documentation to	the extent that such documents are included in the fields searched
	FIGURE - ADDRESSES
Electronic data base consulted during the international search ((name of data base and, where practicable, search terms used)
Medline, Biosis, US Pats on WEST	MOTALIAN IN THE RESERVE OF THE PARTY OF THE
C. DOCUMENTS CONSIDERED TO BE RELEVANT.	CS ASJAVES
Category* Citation of document, with indication, where	appropriate, of the relevant passages Relevant to claim No.
X ABEYDEERA et al. Fertilization and Vitro of Pig. Oocytes Inseminated Medium with Frozen-Thawed Ejacul Reproduction. 1997, Vol. 57, page Methods.	in a Modified Tris-Buffered
	SAU AUD DESCRIPTION DE LA MANTINA DE LA MANTINA DE LA MANTINA DE
OCCUPATE SEVEN	OF DEPTH STAND
The DA PARENT SO TO	
N2 1	STATUS - MECTER SO
Further documents are listed in the continuation of Box	C. See patent family annex.
Special categories of cited documents:	To later document published after the international filing date or priority
"A" document defining the general state of the art which is not considered to be of particular relevance	date and not in conflict with the application but cited to understand the principle or theory underlying the invention
B earlier document published on or after the international filing date	"X" document of particular relevances the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	when the decument is teken along
O document réferring to an oral disclosure, use, exhibition or other	combined with one or more other such doctaments, such combination being obvious to a person skilled in the art
P document published prior to the international filing date but later than the priority date claimed	
Date of the actual completion of the international search	Date of mailing of the international search report
06 APRIL 2000	18 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SANDRA SAUCIER Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT International application No.

International application No. PCT/US99/28408

	smational proof has not been established in respect of contried
	mational report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
ſ	Claims Nos.
L	because they relate to subject matter not required to be searched by this Authority, namely:
	in a real state of the second of the real second of the real second of the second of t
	of agram of the experience of any of any of a contract of the second of the contract of the co
	一直 とは 15・1 とは 14と 14 という 12 12 15 15 15 15 15 15 15 15 15 15 15 15 15
	the base of the set the left because the control of
: 1	The form of the first state of the control of the c
F	Claims Nosified and Additional Section 18 18 18 18 18 18 18 18 18 18 18 18 18
Ļ	because they relate to party of the international application that do not comply with the prescribed manifestation that do not comply with the prescribed
	and water the street like international scarch can be carried out specifically.
è · ;	e transport transport et authorite and programment and contract and the contract and the contract and contract
•	,大学 \$P\$ \$P\$ \$P\$ \$P\$ \$P\$ \$P\$ \$P\$ \$P\$ \$P\$ \$P
	and the first of t
Г	Claims Nos.:
_	because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
įÌ	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)
s h	national Searching Authority found multiple inventions in this international application, as follows:
	可以投入,更加强,更是在1900年的,但是一个人,是一个人,是一个人,是一个人,是一个人,他们们的一个人,不是一个人,不是一个人,不是一个人,不是一个人,不是一
÷	ass Sec Extra Sheets should be the transfer of the second
; : ,	ind the transfer of the bottom by the transfer of the control of t
:::	
	के पित्र के इस अपने क्षेत्र के के के किस के किस किस के किस क
	प्रस्तिकेक भीत्र स्थापक केंद्र क्षेत्र क्षेत्र का त्रा किस्सार है। इस स्थापक केंद्र का किस्सार के किस के किस क इस स्थापक केंद्र के किस के
	प्रस्तिकेक भीत्र स्थापक केंद्र क्षेत्र क्षेत्र का त्रा किस्सार है। इस स्थापक केंद्र का किस्सार के किस के किस क इस स्थापक केंद्र के किस के
	प्रस्तिकेक भीत्र स्थापक केंद्र क्षेत्र क्षेत्र का त्रा किस्सार है। इस स्थापक केंद्र का किस्सार के किस के किस क इस स्थापक केंद्र के किस के
	प्रस्तिकेक भीत्र स्थापक केंद्र क्षेत्र क्षेत्र का त्रा किस्सार है। इस स्थापक केंद्र का किस्सार के किस के किस क इस स्थापक केंद्र के किस के
	प्रस्तिकेक भीत्र स्थापक केंद्र क्षेत्र क्षेत्र का त्रा किस्सार है। इस स्थापक केंद्र का किस्सार के किस के किस क इस स्थापक केंद्र के किस के
	Andrew State (State State Stat
	प्रस्तिकेक भीत्र स्थापक केंद्र क्षेत्र क्षेत्र का त्रा किस्सार है। इस स्थापक केंद्र का किस्सार के किस के किस क इस स्थापक केंद्र के किस के
	As all required additional search fees were timely paid by the applicant, this international search report covers all search
	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite page
	As all required additional search fees were timely paid by the applicant, this international search report covers all search
-	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payers any additional fee.
	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payers any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report co
	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payers any additional fee.
	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payers any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report co
	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payers any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report co
	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payers any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report co
	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payers any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report co
	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payers any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report co
	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payers any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report co
	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payof any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report couly those claims for which fees were paid, specifically claims Nos.:
×	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payer of any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report could be required additional search specifically claims Nos.:
x)	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payof any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report couly those claims for which fees were paid, specifically claims Nos.:
x	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payer of any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report could be required additional search specifically claims Nos.:
x	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payer of any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report could be required additional search specifically claims Nos.:
<u>x</u>]	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payer of any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report could be required additional search specifically claims Nos.:
X)	As all required additional search fees were timely paid by the applicant, this international search report covers all search claims. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payer of any additional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report could be required additional search specifically claims Nos.:

INTERNATIONAL SEARCH REPORT

International application No. PCT/US99/28408

Company of the comment of the comment of the second

II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING	entropies a la la participa de la manda de la companya de la companya de la companya de la companya de la comp
ISA found multiple inventions as follows:	
and the second s	nging gage in grimmagan sammagan sammagan bilan sammagan sammagan sammagan sammagan sammagan sammagan sammagan Ang managan sammagan
The second secon	e lead has been been been become in the
analization contains claims directed to more than one species of the generic inver	ntion. These species are deemed to
Unity of Invention because they are not so linked as to form a single inventive of	oncebt anger LCT Whie 13.11 m
for more than one species to be searched, the appropriate additional search fees	must be paid. The species are as
ws:	
each stage claimed in the method is a distinct species, such as the species of the	stage of claim 2, the species of
topo of claim 10 of claim 16 of claim 22, of claim 25, of claim 36, of claim 43,	and the stage of claim 34.
are each medium is a distinct species. For example, the species of the stage of C	laim 2 has distinct species of
from one of claims 3-0 (7 species). The species of the stage of claim 10 has the	be media of the species of claims
5 (4 species). The species of the stage of claim 16 has the species of the media cies of the stage of claim 22 has the media of the species of claims 23 and 24 (2.5	species). The species of the stage
aim 25, has the media of the species of claims 27.35 (9 species). The species of	the stage of claim 36 has the
of the energies of claims 32.42 (5 species). The species of the stage of claim 4.	7 DER IDE MEGIE SPECIES OF CIRILIE
nd 46 (2 species). The species of the stage of claim 54 has the media of the spec	cies of claims 33-00 (6 species).
is a total of 40 distinct species of stages of claim 1 and the media of the dependent	ent claims.
following claim is generic: claim 1.	Course Magn
and the second of the private supplies with an experience of the second of the second	ति हेप्परकेत्या चार र इस स्वर्गिय करणका । ६ 💮
ial technical feature because it is well known in the art to modify the media while	procedure, Forgexample, policine ubated in NCSU medium without
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuted then the fertilized oocytes were incubated in NCSU medium with BSA as taught to be been medium and would have the same "core salts" throughout the process. The	procedure. For example, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used
which fulfills the limitation recited as "a core group" between stages of an IVF (the cumulus complexes were incubated in NCSU medium containing FF, then incubated the fertilized pocytes were incubated in NCSU medium with BSA as taught	procedure. For example, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuted then the fertilized oocytes were incubated in NCSU medium with BSA as taught to be been medium and would have the same "core salts" throughout the process. The	procedure. For example, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuted then the fertilized oocytes were incubated in NCSU medium with BSA as taught to be been medium and would have the same "core salts" throughout the process. The	procedure. For example, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuted then the fertilized oocytes were incubated in NCSU medium with BSA as taught to be been medium and would have the same "core salts" throughout the process. The	procedure. For example, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuted then the fertilized oocytes were incubated in NCSU medium with BSA as taught to be been medium and would have the same "core salts" throughout the process. The	procedure. For example, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuted the fertilized oocytes were incubated in NCSU medium with BSA as taught the base medium and would have the same "core salts" throughout the process. The ial technical feature, and therefore, lacks unity of invention.	procedure, Por _e example, porcine ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuted then the fertilized oocytes were incubated in NCSU medium with BSA as taught to be been medium and would have the same "core salts" throughout the process. The	procedure, Por _e example, porcine ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuted then the fertilized oocytes were incubated in NCSU medium with BSA as taught the base medium and would have the same "core salts" throughout the process. The later hand therefore, lacks unity of invention.	procedure, rogerampie, governo ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuted the fertilized oocytes were incubated in NCSU medium with BSA as taught the base medium and would have the same "core salts" throughout the process. The ial technical feature, and therefore, lacks unity of invention.	procedure, rogerampie, governo ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuthen the fertilized oocytes were incubated in NCSU medium with BSA as taught to base medium and would have the same "core salts" throughout the process. The ial technical feature, and therefore, lacks unity of invention.	procedure, Porcesample, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a season to exist the same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the same the same to same the same
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuthen the fertilized oocytes were incubated in NCSU medium with BSA as taught to base medium and would have the same "core salts" throughout the process. The ial technical feature, and therefore, lacks unity of invention.	procedure, Porcesample, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a season to exist the same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the same the same to same the same
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuted then the fertilized oocytes were incubated in NCSU medium with BSA as taught the base medium and would have the same "core salts" throughout the process. The later hand therefore, lacks unity of invention.	procedure, Porcesample, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a season to exist the same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the same the same to same the same
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuthen the fertilized oocytes were incubated in NCSU medium with BSA as taught to base medium and would have the same "core salts" throughout the process. The ial technical feature, and therefore, lacks unity of invention.	procedure, Porcesample, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a season to exist the same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the same the same to same the same
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuthen the fertilized oocytes were incubated in NCSU medium with BSA as taught to base medium and would have the same "core salts" throughout the process. The ial technical feature, and therefore, lacks unity of invention.	procedure, Porcesample, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a season to exist the same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the same the same to same the same
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuthen the fertilized oocytes were incubated in NCSU medium with BSA as taught to base medium and would have the same "core salts" throughout the process. The ial technical feature, and therefore, lacks unity of invention.	procedure, Porcesample, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a season to exist the same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the same the same to same the same
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuthen the fertilized oocytes were incubated in NCSU medium with BSA as taught to base medium and would have the same "core salts" throughout the process. The ial technical feature, and therefore, lacks unity of invention.	procedure, Porcesample, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a season to exist the same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the same the same to same the same
which fulfills the limitation recited as "a core group" between stages of an IVF of the cumulus complexes were incubated in NCSU medium containing FF, then incuthen the fertilized oocytes were incubated in NCSU medium with BSA as taught to base medium and would have the same "core salts" throughout the process. The ial technical feature, and therefore, lacks unity of invention.	procedure, Porcesample, policine ubated in NCSU medium without by Abeydeera et al. NCSU is used he process as claimed lacks a season to exist the same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the state for each in each of the same to same the same the same to same the same

200 新港区 5 4 6 E

Transfer Street ज्ञास्त्र का क

7 / 212 t <u>1</u>

AND THE STANDING OF THE Mediana cosastor 28 and CAR Hotel 1377 St. 13 5048 St. 3 1. 34 PM 电影 10 1

> IF THE REPORT OF THE SERVICE AND 3 79 3 3 40

> > The ASSESSMENT OF SAIDS A

COUNTRY CHEST HARRIST .

1,060 A , VI and J

- Acte or wat halphening on the BAR TO HERE ELECTION FOR THE

ार्याः चौत्रश्रीयम्बद्धः अद्याद्यने कान्यक्षेत्रस्य ।

(Man Serie 在175 日付き 1750 日本の計

to 2011 the father and the region of

·通过10年8月1日 南州中央1906年日

4040004X864043

The Board State of the Com-

·33 31.

1931 - 1 英国 1 Yant.

CONTRACTOR

1:10

A Section 15 and the first of the first of the second

THIS PAGE BLANK (USPTO)

How March Charles were to be good to good Que and a supply of the company of the figure servative to senser in the flooright and in the left State of the state note our motive and lead and array with making instables ou discuss a los years peachuriges is not oug The Mark Commission and Commission of the Commis teschied in the first consumers of the god god Alexander go garage to grant to grant the And the state of t Comments of the Comments of th loafe Table with the major man ... 1 The off reserve with a will as a comment THE ROY OF LINE OF THE STREET OF THE STREET, AND STREE The second of th CV 9 17 Sarot Cerean Barto to to to. AND PRODUCED A CONTRACT OF THE BY THE CONTROL OF A BLOWN, I WARRED TO SHE WILL

- 11 ٠.; .: Γ...

1. 11